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<b>(21) International Application Number:</b> PCT/AU98/00812 <b>(22) International Filing Date:</b> 25 September 1998 (25.09.98) <b>(30) Priority Data:</b> PO 9479 26 September 1997 (26.09.97) AU <b>(71) Applicant (for all designated States except US):</b> COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DENNIS, Elizabeth, Salisbury [AU/AU]; 100 Hopetoun Circuit, Yarralumla, ACT 2600 (AU). DOLFERUS, Rudi, Alfons, Marie [BE/AU]; 56 Maribymong Avenue, Kaleen, ACT 2617 (AU). HOEREN, Frank, Ullrich [DE/AU]; 56 Duffy Street, Ainslie, ACT 2602 (AU). PEACOCK, William, James [AU/AU]; 16 Brassey Street, Deakin, ACT 2600 (AU). <b>(74) Agents:</b> SLATTERY, John, Michael et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD OF REGULATING GENE EXPRESSION  <b>(57) Abstract</b>  The present invention provides genetic constructs and methods for altering the expression of one or more environmental stress-related genes in plant cells. The invention further provides methods of modifying the expression of particular stress-related genes in plants in response to environmental stresses, including anaerobic stress, dehydration stress and cold stress.		

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## METHOD OF REGULATING GENE EXPRESSION

### FIELD OF THE INVENTION

The present invention relates generally to reagents and methods for altering gene expression.

- 5 More particularly, the invention provides genetic constructs and methods for altering the expression of one or more environmental stress-related genes in plant cells. The invention provides the means by which a plant cells response to a wide range of environmental stresses may be modified.

### 10 GENERAL

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

- This specification contains nucleotide and amino acid sequence information prepared using  
15 the programme PatentIn Version 2.0, presented herein after the bibliography. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210> 1, <210> 2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields  
20 <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (eg. <400> 1, <400> 2, etc).

- The designation of nucleotide residues referred to herein are those recommended by the  
25 IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine,  
30 V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine

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and N represents any nucleotide residue.

The designations for amino acid residues referred to herein are set forth in Table I.

- 5 As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular source albeit not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion  
10 of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of steps or elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that  
15 the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions or compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps, features, compositions or compounds.

## 20 BACKGROUND TO THE INVENTION

Plants are exposed to a wide range of environmental and physiological stresses which lead to reduced productivity and in severe or prolonged situations, cell death may result leading to crop losses. For example, anaerobic stress, flooding, drought, heat, cold and salinity are all common environmental stresses to which plants are exposed and which are capable of  
25 reducing plant productivity. The production of plants, in particular agricultural and horticultural crop plants, which have improved tolerance to such environmental stresses is clearly desirable.

Plants may respond to environmental stress by altering cellular metabolism as part of an  
30 adaptive stress response, presumably by inducing the expression of specific proteins and/or

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down-regulating the expression of other proteins. However, for most environmental stresses the nature of the adaptive stress response is, at best, incompletely characterised. In particular, the full complement of proteins induced by any environmental stress is not known.

- 5 Plants respond to drought stress by inducing the expression of a class of genes known as "dehydrins", at least by increasing transcription of the dehydrin genes (Close *et al.*, 1989). However, the precise function of the dehydrins is unknown.

The response to anaerobic stress, such as occurs during flooding, is one of the best-  
10 characterised stress responses of plants, at the physiological and biochemical levels. Plants respond to anaerobic stress by switching from oxidative phosphorylative respiration to fermentative glycolysis, by suppressing normal protein synthesis and inducing the synthesis of so-called "anaerobic polypeptides" (ANPs; Sachs *et al.*, 1980, Sachs *et al.*, 1996). The ANPs are expressed in several different tissues of anaerobically-stressed plants (Okimoto *et al.*, 1980). These ANPs may be classified into at least four functional groups: (i) Glucose-  
15 mobilising enzymes, for example sucrose synthase, amongst others; (ii) stem-glycolytic enzymes including phosphoglucumutase, phosphoglucoisomerase, fructose-1,6-diphosphate aldolase, glyeraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase and enolase, amongst others; (iii) fermentative enzymes including pyruvate decarboxylase and alcohol  
20 dehydrogenase, amongst others; and (iv) amino acid biosynthetic enzymes, for example alanine amino transferase, amongst others. The induction of pyruvate decarboxylase and alcohol dehydrogenase is believed to be of particular significance to survival of the plant, because the regeneration of NAD<sup>+</sup> facilitated by these enzymes may enable submerged plant roots to continue anaerobic glycolysis for prolonged periods. In the absence of a functional  
25 alcohol dehydrogenase-1 (*Adh1*) gene, anaerobically-stressed or flooded maize roots reduce pyruvate to lactate, resulting in cytoplasmic acidification, irreversible cell damage and death.

Elements in the promoter of the maize *ADHI* gene, which appear to be required for anaerobic induction of gene expression, have been identified (Walker *et al.*, 1987; Olive *et al.*, 1990;  
30 1991a, b). The Anaerobic Response Element (ARE) lies between -100 and -140 relative to

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the transcription start and is a bipartite element with two copies of a GT-element (5'-[T/C]GGTTT-3'), and two GC-elements (5'-GCC[G/C]C-3'). The GC elements bind a GC-Binding Protein (GCBP-1; Olive *et al.*, 1991b) and both appear to be critical for expression, however the nature of the GC-Binding Protein has yet to be elucidated. The  
5 GT-motifs are also critical for anaerobic induction and expression (Walker *et al.*, 1987) and are "footprinted" in vivo by dimethyl sulfate (Ferl and Nick, 1987), suggesting proteins bind to the GT-motifs. No GT binding protein has been identified.

*Arabidopsis ADHI* is a gene induced by anaerobic conditions and a number of other  
10 environmental stimuli (low temperature, dehydration) and the phytohormone ABA (Dolferus *et al.* 1994; de Bruxelles *et al.*, 1996). The *Arabidopsis ADHI* promoter contains sequences similar to the ARE between -160 and -140, with the GT-motifs in the opposite orientation compared to the maize *Adhl* promoter (GT-motif: 5'-AAACCAA-3'; GC-motif: 5'-GCCCC-3'). Functional analysis in transgenic *Arabidopsis* plants showed both motifs to  
15 be critical for *ADHI* induction by low oxygen and by other stress conditions (Dolferus *et al.*, 1994). The *Arabidopsis* GC-motif, together with two upstream elements between positions -230 to -219, and -197 to -174, were shown, by footprinting, to be contact points for proteins (Ferl and Laughner, 1989). The former region contains the G-box-1 sequence (5'-CCACGTGG-3'; -226 to -219), which was shown to be required for the low temperature,  
20 dehydration and ABA responses (Dolferus *et al.*, 1994; de Bruxelles *et al.*, 1996). Mutagenesis of portion of the G-box-2 sequence (5'-CCAAGTGG-3'), in the second footprint region, did not have clearcut effects on *ADHI* expression (Dolferus *et al.*, 1994; de Bruxelles *et al.*, 1996).

25 Notwithstanding that different stress responses may each produce an adaptive stress response, there appears to be limited nexus between responses to different stress situations, making a single solution to improving the tolerance of any plant species to several environmental stress situations a difficult task. Additionally, different plant species appear to mount different responses to the same environmental stress, for example by inducing stress-related genes in  
30 different tissues and/or by inducing different stress-related genes. Clearly, a single solution

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to improving the stress tolerance of several plant species to any one environmental stress is not a straightforward procedure.

Moreover, the absence of knowledge in relation to the precise mechanisms involved in  
5 regulating the adaptive stress responses of plants, in particular the transcription factors and second-messengers involved, further complicates the task of producing stress tolerant plants.

### SUMMARY OF THE INVENTION

In work leading up to the present invention, the inventors sought to elucidate the nature of  
10 transcription factors involved in regulating the expression of different *Adh1* genes in plants, in response to anaerobic stress and cold stress. The inventors have demonstrated that a family of transcription factors, the MYB2 family of proteins, are capable of inducing the expression of stress-related genes in plant cells, in response to several stress situations. The regulation of stress-related gene expression by the MYB2 family of proteins provides the means for  
15 producing plants with improved tolerance to a wide-range of environmental stresses.

Accordingly, one aspect of the present invention provides a method of modifying the expression of a stress-related gene in a cell, said method comprising expressing in said cell a MYB2 protein or a homologue, analogue or derivative thereof for a time and under  
20 conditions sufficient for expression of said stress-related gene to be increased, reduced or otherwise altered.

Whilst not limiting the scope of the present invention, the MYB2 protein of the present invention is particularly useful for the purposes of inducing or repressing the expression of  
25 plant-expressible genes which are involved in the plant's response to anaerobic stress, flooding stress, cold stress, dehydration stress, drought stress, heat stress or salinity, amongst others, for example those genes selected from the list comprising sucrose synthase, phosphoglucomutase, phosphoglucose isomerase, fructose-1,6-diphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, enolase, pyruvate  
30 decarboxylase, alcohol dehydrogenase and alanine amino transferase, amongst others.

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A further aspect of the invention provides a genetic construct when used to express a MYB2 protein in a cell to modify the expression of a stress-related gene in said cell, preferably a gene which encodes an anaerobic polypeptide (ANP).

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A further aspect of the invention provides a method of producing a stress-tolerant plant comprising expressing a MYB2 protein or a homologue, analogue or derivative thereof in said plant for a time and under conditions sufficient for expression of a stress-related gene in said plant to be increased, reduced or otherwise altered.

10

In an alternative embodiment, the invention provides a method of producing a stress-tolerant plant comprising the steps of transforming plant tissue or cells with a genetic construct which at least comprises a nucleotide sequence which encodes a MYB2 protein placed operably under the control of a plant-expressible promoter sequence, regenerating said tissue or cells  
15 into a whole plant and expressing said MYB2 protein in said plant for a time and under conditions sufficient for expression of a stress-related gene in said plant to be increased, reduced or otherwise altered.

The present invention provides, in a further aspect, transgenic plants, plant cells and tissues,  
20 which comprise a genetic construct as described *supra*. Preferably, said transgenic plant, cell or tissue has modified expression of a stress-related gene. More preferably, said transgenic plant, cell or tissue is stress-tolerant.

#### BRIEF DESCRIPTION OF THE DRAWINGS

25 **Figure 1-1** is a schematic representation of the *Arabidopsis thaliana* and maize *ADH1* promoter sequences, comparing their functional organisation. Arrows indicate the orientation of the GT-motifs (GT-1, GT-2) and GC-motifs (GC-1, GC-2) in both promoters. The positions of the G-Box elements (G-Box-1, G-Box-2) in the *A. thaliana ADH1* promoter are also indicated. The positions of the two Myb-binding sites (MBS-1 and MBS-2) are also  
30 indicated. Numbering above the sequences refers to the positions of nucleotides, relative to

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the start of transcription of each gene. At the bottom of the Figure is a representation of the consensus sequences of MYB-binding sites derived from the genes of both animals (left) and plants (right).

5 **Figure 1-2** is a schematic representation of the nucleotide sequences of the MBS-1 and MBS-2 oligonucleotides which comprise the wild-type MBS-1 and MBS-2 binding sites, respectively in the *Arabidopsis thaliana ADH1* promoter sequence. The nucleotide sequences of oligonucleotides having mutations in the region of the putative MBS-1 binding site ( $\Delta$ MBS-1/1;  $\Delta$ MBS-1/2) and the putative MBS-2 binding site ( $\Delta$ MBS-2), in addition to an  
10 oligonucleotide comprising multiple repeats of the MBS-1 binding site (4xMBS-1), are also indicated. The nucleotide sequences of oligonucleotides described by Dolferus *et al.* (1994) are also indicated ( $\Delta$ GT,  $\Delta$ GC,  $\Delta$ GBOX1,  $\Delta$ GBOX2). Underlined nucleotides in the MBS-1, MBS-2 and 4xMBS-1 sequences are those which have been mutated in the corresponding mutant sequences. Underlined nucleotides in italics are mutated compared to the wild type  
15 sequence. Double stranded equivalents of these oligonucleotides were used in gel retardation experiments.

**Figure 2-1** is- a copy of a photographic representation of a gel retardation autoradiograph showing the binding of purified GST-AtMYB2 fusion protein to radioactively labelled MBS-1  
20 and MBS-2 oligonucleotides (lanes marked 0). Binding is reduced by the addition of unlabelled competitor DNA of the same sequence as the labelled probe (lanes marked 10, 100), but not by the addition of non-specific oligonucleotides (lanes marked NS). Lanes marked FP indicate reaction mixtures containing the labelled probes without added fusion protein. Numbers on the top of each figure indicate fold molar excess of cold competitor.

25

**Figure 2-2** is a copy of a photographic representation of a gel retardation autoradiograph showing the increased binding of purified GST-AtMYB2 fusion protein to radioactively labelled 4xMBS-1 oligonucleotides, compared to MBS-1 oligonucleotides as indicated by the higher molar excess of unlabelled competitor DNA that is required to eliminate binding.  
30 Binding of fusion protein to MBS-1 and 4XMBS-1 oligonucleotides was carried out in the

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presence (lanes marked 10, 100, 200, 500 ) of unlabelled competitor DNA of the same sequence as the labelled probe or alternatively, in the absence of unlabelled competitor DNA (lanes marked 0). Numbers on the top of each figure indicate fold molar excess of cold competitor. Lanes marked FP indicate reaction mixtures containing the labelled probes  
5 without added fusion protein.

Figure 2-3 is a copy of a photographic representation of three gel retardation autoradiographs showing the increased binding of purified GST-AtMYB2 fusion protein to radioactively labelled MBS-1 oligonucleotide (left and right panels) and radioactively-labelled MBS-2  
10 oligonucleotide (middle panel), in the absence of unlabelled competitor DNA (lanes marked 0 in each panel) or in the presence of varying amounts of unlabelled MBS-1 (left panel, lanes marked 10 and 100), MBS-1/1 (left panel, lanes marked 10, 100 and 500), MBS-1/2 (right panel, lane marked 100) or MBS-2 (middle panel, lane marked 100) competitor oligonucleotide. Numbers on the top of each figure indicate fold molar excess of cold  
15 competitor. Lanes marked FP indicate reaction mixtures containing the labelled probes without added fusion protein. Data indicate that the binding of AtMYB2 to MBS-1 and MBS-2 can not be competed by competitor oligonucleotides with a mutated AAC core sequence ( $\Delta$ MBS-1/1 and  $\Delta$ MBS-2 respectively), however the mutation of the AAC core to GAC ( $\Delta$ MBS-1/2 oligonucleotide), did not completely eliminate the capacity of the oligonucleotide  
20 to compete with labelled wild type MBS-1 oligonucleotide for binding.

Figure 3-1 is a copy of a photographic representation showing the induction of *ADH1* gene expression (top panel) and AtMYB2 gene expression (lower panel) by stress treatments. C: control; AN: low oxygen treatment; D: dehydration; CD: low temperature; ABA: ABA  
25 treatment. AtMYB2 is induced by all stress treatments, and expression is higher in roots (solid black bars) than in shoots (hatched bars). Error bars represent standard errors for three repeats.

Figure 3-2 is a copy of a photographic representation of a northern hybridisation showing the

kinetics of AtMYB2 mRNA accumulation in the leaves (L) and roots (R) of plants, after 0 hours (0 H), 2 hours (2 H), 4 hours (4 H), 6 hours (6 H) and 8 hours (8 H) of low oxygen stress treatment. Accumulation of mRNA is preferentially in the roots of the plant.

5 Figure 3-3 is a copy of a graphical representation showing the induction kinetics of AtMYB2 mRNA ( $\square$ ), compared to *ADH1* mRNA accumulation ( $\circ$ ), in *Arabidopsis* root cultures, under conditions of low oxygen stress (top panel), dehydration stress (second-to-top panel), low temperature stress (second-to bottom panel) and in the presence of abscisic acid (ABA; bottom panel). Results are expressed as fold induction compared with mRNA levels at the  
10 start of the treatment.

Figure 4 is a copy of a graphical representation showing the induction kinetics of AtMYB2 ( $\blacksquare$ ) mRNA under low oxygen conditions, compared to the kinetics of induction of the *ADH1* ( $\blacklozenge$ ), *Arabidopsis thaliana* pyruvate decarboxylase (*PDC1*;  $\blacktriangle$ ) and *Arabidopsis thaliana*  
15 sucrose synthase (*ASUS1*;  $\bullet$ ) genes. Results are expressed as fold induction compared with mRNA levels at the start of the treatment. The expression of the *PDC1* and *ASUS1* genes is root-specific.

Figure 5 is a copy of a photographic representation showing the expression of the genetic  
20 constructs CADH-GUS (left panels) and 35S-GUS (right panels) in pea leaves. The expression of the CADH-GUS construct following co-transfection of pea leaves with the construct 35S-AtMYB2 is also indicated (middle panels). The increased spot size and intensity of staining in the samples which were co-transfected with CADH-GUS and 35S-AtMYB2, indicates that AtMYB2 is able to transactivate *ADH1*-promoter activity.

25

Figure 6-1 is a copy of a graphical representation showing AtMYB2 transactivation of an ADH-GUS construct in *Arabidopsis thaliana* mesophyll protoplasts and *N. plumbaginifolia* suspension protoplasts. The ADH-GUS constructs indicated on the x-axis were expressed in protoplasts in the absence (hatched bars) or the presence (solid black bars) of the construct  
30 35S-AtMYB2. In control reactions, the construct 35S-GUS was assayed. Transient assays

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using *A. thaliana* mesophyll protoplasts indicate that AtMYB2 is able to transactivate *ADH1*-promoter-driven GUS expression by a factor 2-2.5. This was confirmed also by using protoplasts of *N. plumbaginifolia* suspension cells, where higher transactivation of about 2.5-4.5-fold were consistently observed.

5

Figure 6-2 is a copy of a graphical representation showing AtMYB2 transactivation of the wild-type *ADH1* promoter (*ADH1*-GUS) and several substitution mutant promoter constructs containing mutations in the GT-1 (MBS-1) motif ( $\Delta$ GT), GC-1 motif ( $\Delta$ GC), G-Box-1 motif ( $\Delta$ Gbox1), G-Box-2 motif ( $\Delta$ Gbox2) or the MBS-2 motif ( $\Delta$ MBS2) in *Arabidopsis thaliana* mesophyll protoplasts and *N. plumbaginifolia* suspension protoplasts. The mutations in each of the mutant constructs are indicated in Figure 1-2. The *ADH*-GUS constructs were expressed in protoplasts in the absence (hatched bars) or the presence (solid black bars) of the construct 35S-AtMYB2. Data indicate that AtMYB2 is not able to transactivate *ADH*-GUS expression when the GT-motif (MBS1 motif) is mutated ( $\Delta$ GT), but activation is not affected when MBS-2 is mutated ( $\Delta$ MBS2). Transactivation potential is reduced when the GC-motif, the G-box-1 motif or the G-box-2 motif is mutated ( $\Delta$ GC,  $\Delta$ Gbox1 and  $\Delta$ Gbox2, respectively).

Figure 7-1 is a copy of a graphical representation of northern blot hybridisation data showing *ADH1* mRNA accumulation under conditions of anaerobic stress (AN), dehydration, (D), cold stress (CD) and abscisic acid treatment (ABA) in four-weeks-old *Arabidopsis thaliana* plantlets, compared to untreated plants (C), in the absence (filled bars) or presence (stippled bars) of 10  $\mu$ M cycloheximide (CHX).

Figure 7-2 is a copy of a graphical representation of linear RT-PCR data showing the effect of cycloheximide on AtMYB2 expression after anaerobic stress (AN), dehydration for four hours (D/4H) or ten hours (D/10H), cold stress for four hours (CD/4H) or 24 hours (CD/24H) and abscisic acid treatment (ABA) in four-weeks-old *Arabidopsis thaliana* plantlets, compared to untreated plants (C), in the absence (filled bars) or presence (stippled bars) of 10  $\mu$ M cycloheximide (CHX). and ABA treatment. RNA from plants subjected to

dehydration (D) or low temperature (CD) was extracted at the 2 time-points showing maximal induction (see Figure 3-3).

Figure 8 is a schematic representation of a model for the functional organisation of the *Arabidopsis ADH1* promoter, showing regulation of the promoter by AtMYBs and environmental stresses. The *cis*-acting motifs shown in Figure 1-1 are also indicated, together with proteins which may bind to these regions.

Figure 9 is a schematic representation of plasmid pCMYB/5', containing the 5' RT-PCR fragment of the AtMYB cDNA, from the *Bam*HI site upstream of the ATG to an internal *Xho*I site. The position of the ampicillin-resistance gene (Amp) is also indicated. Restriction enzyme sites for the enzymes *Sac*I, *Bst*XI, *Sac*II, *Eag*I, *Not*I, *Xba*I, *Spe*I, *Bam*HI, *Nco*I, *Xho*I, *Dra*II, *Apa*I and *Kpn*I are also indicated in the Figure.

Figure 10 is a schematic representation of plasmid pCMYB/3', containing the 3' RT-PCR fragment of the AtMYB cDNA, from the *Bam*HI site at the end of the cDNA to an internal *Xho*I site. The position of the ampicillin-resistance gene (Amp) is also indicated. Restriction enzyme sites for the enzymes *Sac*I, *Bst*XI, *Sac*II, *Eag*I, *Not*I, *Xba*I, *Spe*I, *Bam*HI, *Nco*I, *Xho*I, *Dra*II, *Apa*I and *Kpn*I are also indicated in the Figure.

20

Figure 11 is a schematic representation of plasmid pCATMYB2/S, containing the spliced 5' RT-PCR and 3' RT-PCR fragments derived from clones pCMYB/5' and pCMYB/3', respectively. The position of the ampicillin-resistance gene (Amp) is also indicated. Restriction enzyme sites for the enzymes *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Pst*I, *Sph*I and *Hind*III are also indicated in the Figure.

25

Figure 12 is a schematic representation of the plasmid p35S-cAtmyb2/S, which comprises the CaMV 35S promoter sequence (P 35S) operably in connection with the AtMYB2 structural gene sequence (cAtMyb2) in the sense orientation and placed upstream of the octopine synthase gene terminator (3' OCS). The position of the ampicillin-resistance gene

30

(Amp) is also indicated. Restriction enzyme sites for the enzymes *NotI*, *XhoI*, *EcoRI*, *KpnI*, *SmaI*, *ClaI*, *HindIII*, *BamHI* and *XbaI* are also indicated in the Figure.

Figure 13: Schematic representation of the plasmid vector pCADH-GUS, which comprises the *Arabidopsis thaliana ADHI* promoter sequence (PAdh) operably in connection with the bacterial *uidA* gene (GUS) in the sense orientation and placed upstream of the nopaline synthase gene terminator (NOS). Restriction enzyme sites for the enzymes *EcoRI*, *SmaI*, *KpnI*, *SacI*, *BamHI*, *NcoI*, *XbaI*, *SalI*, *PstI*, *SphI* and *HindIII* are also indicated in the Figure. ATG indicates the translation start site of the *uidA* gene.

10

Figure 14 is a schematic representation of plasmid pCATMYB2(251), containing a 3'-truncated AtMYB cDNA. The position of the ampicillin-resistance gene (Amp) is also indicated. Restriction enzyme sites for the enzymes *EcoRI*, *SacI*, *KpnI*, *SmaI*, *BamHI*, *XbaI*, *SalI*, *PstI*, *SphI* and *HindIII* are also indicated in the Figure.

15

Figure 15 is a schematic representation of plasmid pCATMYB2(207), containing a 3'-truncated AtMYB cDNA. The position of the ampicillin-resistance gene (Amp) is also indicated. Restriction enzyme sites for the enzymes *EcoRI*, *SacI*, *KpnI*, *SmaI*, *BamHI*, *XbaI*, *SalI*, *PstI*, *SphI* and *HindIII* are also indicated in the Figure.

20

Figure 16 is a schematic representation of the plasmid p35S-CATMYB2(251), which comprises the CaMV 35S promoter sequence (P 35S) operably in connection with the 3'-truncated AtMYB2 structural gene sequence cAtMyb2( $\Delta$ 251) in the sense orientation and placed upstream of the octopine synthase gene terminator (3' OCS). The position of the ampicillin-resistance gene (Amp) is also indicated. Restriction enzyme sites for the enzymes *NotI*, *XhoI*, *EcoRI*, *KpnI*, *SmaI*, *ClaI*, *HindIII*, *BamHI* and *XbaI* are also indicated in the Figure.

Figure 17 is a schematic representation of the plasmid p35S-CATMYB2(207), which comprises the CaMV 35S promoter sequence (P 35S) operably in connection with the 3'-

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truncated AtMYB2 structural gene sequence cAtMyb2( $\Delta$ 207) in the sense orientation and placed upstream of the octopine synthase gene terminator (3' OCS). The position of the ampicillin-resistance gene (Amp) is also indicated. Restriction enzyme sites for the enzymes *NotI*, *XhoI*, *EcoRI*, *KpnI*, *SmaI*, *ClaI*, *HindIII*, *BamHI* and *XbaI* are also indicated in the

5 Figure.

Figure 18 is a schematic representation of the binary vector pTi 35S-cAtmyb2/S, which comprises the CaMV 35S promoter sequence (P 35S) operably in connection with the AtMYB2 structural gene sequence (cAtMyb2) in the sense orientation and placed upstream

10 of the octopine synthase gene terminator (3' OCS). A plant-expressible chimeric selectable marker gene comprising the nopaline synthase gene promoter operably in connection with the neomycin phosphotransferase structural gene placed upstream of the nopaline synthase terminator (PNOS-NPTII-NOS3') is also indicated. The positions of the Ti plasmid left border (TL) and right border (TR) sequences are also indicated flanking the chimeric genes.

15 The position of the Tn7-derived spectinomycin-resistance/ streptomycin-resistance gene (Tn7 SpR/StR) is also indicated. Restriction enzyme sites for the enzymes *NotI*, *XhoI*, *EcoRI*, *KpnI*, *SmaI*, *ClaI*, *HindIII*, *BamHI* and *XbaI* are also indicated in the Figure.

Figure 19-1 is a schematic representation of the binary genetic construct pTA-CATMYB2,

20 showing the relative arrangement of the *Agrobacterium tumefaciens* Ti plasmid left-border (black arrow; LB) and right border (black arrow; RB) sequences; a chimeric gene comprising the CaMV 35S promoter (black arrow; 35S) driving expression of GVG (hatched bar; GVG), placed upstream of the E9 terminator (wave-lined bar; E9); a selectable marker gene comprising the NOS promoter (filled arrow; NOS) driving expression of the hygromycin

25 phosphotransferase gene (hatched box; HPT) placed upstream of the NOS terminator (checkered box; NOS); and a chimeric AtMYB2 gene comprising the glucocorticoid-inducible 6XUAS/-46 chimeric promoter (black arrow) driving expression of a sense-oriented AtMYB2 structural gene (filled bar; CATMYB2) placed upstream of the *rbcs3A* terminator (cross-hatched box; 3A). Restriction enzyme sites for the enzymes *BamHI*, *XhoI*, *EcoRI*,

30 *KpnI*, *SmaI*, *ClaI*, *HindIII*, *XbaI* and *SpeI* are indicated at the top of the Figure. The chimeric

gene constructs are contained in the pTA7002 vector backbone, which contains a kanamycin-resistance gene.

Figure 19-2 is a schematic representation of the binary genetic construct pTA-CATMYB2 (Δ251), showing the relative arrangement of the *Agrobacterium tumefaciens* Ti plasmid left-border (black arrow; LB) and right border (black arrow; RB) sequences; a chimeric gene comprising the CaMV 35S promoter (black arrow; 35S) driving expression of GVG (hatched bar; GVG), placed upstream of the E9 terminator (wave-lined bar; E9); a selectable marker gene comprising the NOS promoter (filled arrow; NOS) driving expression of the hygromycin phosphotransferase gene (hatched box; HPT) placed upstream of the NOS terminator (checkered box; NOS); and a chimeric AtMYB2 gene comprising the glucocorticoid-inducible 6XUAS/-46 chimeric promoter (black arrow) driving expression of a truncated sense-oriented AtMYB2 structural gene which lacks sequences encoding the carboxy-terminal region downstream of amino acid residue 251 (filled bar; CATMYB2), placed upstream of the *rbcS3A* terminator (cross-hatched box; 3A). Restriction enzyme sites for the enzymes *Bam*HI, *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, *Cla*I, *Hind*III, *Xba*I and *Spe*I are indicated at the top of the Figure. The chimeric gene constructs are contained in the pTA7002 vector backbone, which contains a kanamycin-resistance gene.

Figure 19-3 is a schematic representation of the binary genetic construct pTA-CATMYB2 (Δ207), showing the relative arrangement of the *Agrobacterium tumefaciens* Ti plasmid left-border (black arrow; LB) and right border (black arrow; RB) sequences; a chimeric gene comprising the CaMV 35S promoter (black arrow; 35S) driving expression of GVG (hatched bar; GVG), placed upstream of the E9 terminator (wave-lined bar; E9); a selectable marker gene comprising the NOS promoter (filled arrow; NOS) driving expression of the hygromycin phosphotransferase gene (hatched box; HPT) placed upstream of the NOS terminator (checkered box; NOS); and a chimeric AtMYB2 gene comprising the glucocorticoid-inducible 6XUAS/-46 chimeric promoter (black arrow) driving expression of a truncated sense-oriented AtMYB2 structural gene which lacks sequences encoding the carboxy-terminal region downstream of amino acid residue 207 (filled bar; CATMYB2), placed upstream of the

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*rbcS3A* terminator (cross-hatched box; 3A). Restriction enzyme sites for the enzymes *Bam*HI, *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, *Cla*I, *Hind*III, *Xba*I and *Spe*I are indicated at the top of the Figure. The chimeric gene constructs are contained in the pTA7002 vector backbone, which contains a kanamycin-resistance gene.

5

Figure 20 is a schematic representation of the plasmid p35S-cAtmyb2/A, which comprises the CaMV 35S promoter sequence (P 35S) operably in connection with the AtMYB2 structural gene sequence (cAtMyb2) in the antisense orientation and placed upstream of the octopine synthase gene terminator (3' OCS). The position of the ampicillin-resistance gene  
10 (Amp) is also indicated. Restriction enzyme sites for the enzymes *Not*I, *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, *Cla*I, *Hind*III, *Bam*HI and *Xba*I are also indicated in the Figure.

Figure 21 is a schematic representation of the binary vector pTi 35S-cAtmyb2/A, which comprises the CaMV 35S promoter sequence (P 35S) operably in connection with the  
15 AtMYB2 structural gene sequence (cAtMyb2) in the antisense orientation and placed upstream of the octopine synthase gene terminator (3' OCS). A plant-expressible chimeric selectable marker gene comprising the nopaline synthase gene promoter operably in connection with the neomycin phosphotransferase structural gene placed upstream of the nopaline synthase terminator (PNOS-NPTII-NOS3') is also indicated. The positions of the Ti  
20 plasmid left border (TL) and right border (TR) sequences are also indicated flanking the chimeric genes. The position of the Tn7-derived spectinomycin-resistance/ streptomycin-resistance gene (Tn7 SpR/StR) is also indicated. Restriction enzyme sites for the enzymes *Not*I, *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, *Cla*I, *Hind*III, *Bam*HI and *Xba*I are also indicated in the Figure.

25

Figure 22 is a schematic representation of the binary genetic construct pTA-CATMYB2/A, showing the relative arrangement of the *Agrobacterium tumefaciens* Ti plasmid left-border (black arrow; LB) and right border (black arrow; RB) sequences; a chimeric gene comprising the CaMV 35S promoter (black arrow; 35S) driving expression of GVG (hatched bar; GVG),  
30 placed upstream of the E9 terminator (wave-lined bar; E9); a selectable marker gene

comprising the NOS promoter (filled arrow; NOS) driving expression of the hygromycin phosphotransferase gene (hatched box; HPT) placed upstream of the NOS terminator (checkered box; NOS); and a chimeric antisense AtMYB2 gene comprising the glucocorticoid-inducible 6XUAS/-46 chimeric promoter (black arrow) driving expression of a truncated antisense-oriented AtMYB2 structural gene (filled bar; CATMYB2), placed upstream of the *rbcS3A* terminator (cross-hatched box; 3A). Restriction enzyme sites for the enzymes *Bam*HI, *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, *Cla*I, *Hind*III, *Xba*I and *Spe*I are indicated at the top of the Figure. The chimeric gene constructs are contained in the pTA7002 vector backbone, which contains a kanamycin-resistance gene.

10

Figure 23 is a schematic representation of the plasmid pQEA (NH<sub>2</sub>)AtMYB2, which comprises the 3'-end of the AtMYB2 cDNA clone in the plasmid pQE30, downstream of the poly-Histidine tag (6XHis). The position of the ampicillin-resistance gene (Amp) is also indicated.

15

Figure 24 is a schematic representation of the plasmid pKBSΔ (NH<sub>2</sub>)AtMYB2 #29, which comprises the 3'-end of the AtMYB2 cDNA clone from the plasmid pQE (NH<sub>2</sub>)AtMYB2, cloned into the *Bam*HI site of plasmid pKBS (Stratagene). The position of the kanamycin-resistance gene (Km) is also indicated.

20

Figure 25 is a schematic representation of the binary genetic construct pTAA(NH<sub>2</sub>)ATMYB2, showing the relative arrangement of the *Agrobacterium tumefaciens* Ti plasmid left-border (black arrow; LB) and right border (black arrow; RB) sequences; a chimeric gene comprising the CaMV 35S promoter (black arrow; 35S) driving expression of GVG (hatched bar; GVG), placed upstream of the E9 terminator (wave-lined bar; E9); a selectable marker gene comprising the NOS promoter (filled arrow; NOS) driving expression of the hygromycin phosphotransferase gene (hatched box; HPT) placed upstream of the NOS terminator (checkered box; NOS); and a chimeric antisense AtMYB2 gene comprising the glucocorticoid-inducible 6XUAS/-46 chimeric promoter (black arrow) driving expression of a truncated antisense-oriented AtMYB2 structural gene which lacks sequences encoding the

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amino-terminal region of the protein (filled bar; CATMYB2), placed upstream of the *rbcS3A* terminator (cross-hatched box; 3A). Restriction enzyme sites for the enzymes *Bam*HI, *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, *Cla*I, *Hind*III, *Xba*I and *Spe*I are indicated at the top of the Figure. The chimeric gene constructs are contained in the pTA7002 vector backbone, which contains a  
5 kanamycin-resistance gene.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the present invention provides a method of modifying the expression of a  
10 stress-related gene in a cell, said method comprising expressing in said cell a MYB2 protein or a homologue, analogue or derivative thereof for a time and under conditions sufficient for expression of said stress-related gene to be increased, reduced or otherwise altered.

In a preferred embodiment of the invention, the cell in which expression of the stress-related  
15 gene is modified is a plant cell.

As used herein, the term "expression" in relation to a gene or protein shall be taken to refer to the processes of transcription and/or translation and/or other processes involved in regulating the synthesis of a functional polypeptide. Such "other processes" may include for  
20 example (i) processes regulating transcription of a gene such as triple helix formation, DNA:protein complex formation or protein:protein interactions, amongst others; (ii) processes regulating translation of a gene such as RNA splicing, polyribosome assembly or disassembly, ribonucleoprotein complex formation, RNA:protein complex formation or protein:protein interactions amongst others; (iii) post-translational processes such as  
25 glycosylation, peptide splicing, protein folding, compartmentalisation, transport, and turnover amongst others which may regulate activity of a peptide, polypeptide protein or enzyme in a cell.

The invention is not in any way limited by the precise mechanism or steps involved in  
30 modifying expression of a stress-related gene, the only requirement being that a MYB2

protein or a homologue, analogue or derivative thereof is involved at some level. Whilst not being bound by any theory or mode of action, the present inventors have demonstrated that MYB2 proteins at least bind to one or more sequence motifs in the promoter of a stress-related gene, thereby modifying transcription of that gene. Additional regulatory mechanisms  
5 are clearly possible.

The term "stress-related gene" shall be taken to refer to any gene which is regulated directly or indirectly by an environmental stress in a plant cell, including genes for which expression is increased, reduced or otherwise altered.

10

Preferred stress-related genes are those genes the expression of which is either induced or repressed by anaerobic stress, flooding stress, cold stress, dehydration stress, drought stress, heat stress or salinity, amongst others. More preferably, the stress-related gene is a gene the expression of which is either induced or repressed by anaerobic stress, flooding stress or cold  
15 stress. In an even more preferred embodiment, the stress-related gene is a gene which is capable of being induced by anaerobic stress, flooding stress or cold stress.

In a still more preferred embodiment, the stress-related gene is a gene which encodes an ANP selected from the list comprising sucrose synthase, phosphoglucomutase, phosphoglucose  
20 isomerase, fructose-1,6-diphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, enolase, pyruvate decarboxylase, alcohol dehydrogenase and alanine amino transferase, amongst others.

In a particularly preferred embodiment, the stress-related gene is the alcohol dehydrogenase  
25 gene or the pyruvate decarboxylase gene.

In an alternative embodiment, the stress-related gene is a gene which is capable of being trans-activated by the MYB2 polypeptide, by virtue of the presence of at least one copy of a *cis*-acting regulatory sequence, in particular a MBS-1 motif and/or a GC-motif and/or a G-  
30 box-1 motif and/or a G-box-2 motif, in the promoter region of said stress-related gene to

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which the MYB2 polypeptide may bind to facilitate the initiation or enhancement of transcription of said gene.

As used herein, the terms "MBS-1 motif", "GC-motif", "G-box-1 motif" and "G-box-2 motif" are defined with reference to the nucleotide sequences set forth in Figure 1-1, wherein "GC-motif" extends to include both "GC-1" and "GC-2" motifs set forth in Figure 1-1. However, it is to be understood that the nucleotide sequences of such motifs as set forth in Figure 1-1 may comprise additional nucleotides at their 5'-end and/or 3'-end without affecting their function, provided that the "core" nucleotide sequence is present. It is also to be understood that the terms "MBS-1 motif", "GC-motif", "G-box-1 motif" and "G-box-2 motif" extend further to encompass the complementary nucleotide sequences of the nucleotide sequence set forth in Figure 1-1.

Genes possessing such elements include those stress-related genes encoding the enzymes alcohol dehydrogenase, aldolase, glyceraldehyde phosphate dehydrogenase, pyruvate decarboxylase, sucrose synthase and lactate dehydrogenase, amongst others. The present invention therefore extends to the use of MYB transcription factors, in particular AtMYB2, to regulate the expression of these genes.

Those skilled in the art will be aware that by "induction" of expression of a stress-related gene is meant that expression of said gene is enhanced or increased, independent of the actual level of expression either before or after induction has occurred. Similarly, "repression" of expression means that expression is reduced, inhibited or delayed, independent of the level of expression either before or after repression has occurred. Thus, it is not essential for gene expression to be increased from an undetectable or zero level to qualify as induction or alternatively, to be reduced to an undetectable level to qualify as repression, the only requirement being that the level of expression is detectably altered.

The term "stress" as used herein refers to any alteration in cellular metabolism, reduced cell growth or cell death which is produced by an environmental stressor or by a hormone, second

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messenger or other molecule which is related to or induced by said stressor.

The term "anaerobic stress" means any reduction in oxygen levels sufficient to produce a stress as hereinbefore defined, including hypoxia and anoxia.

5

The term "flooding stress" refers to any stress which is associated with or induced by prolonged or transient immersion of a plant, plant part, tissue or isolated cell in a liquid medium such as occurs during monsoon, wet season, flash flooding or excessive irrigation of plants, etc.

10

"Cold stress" and "heat stress" are stresses induced by temperatures which are respectively, below or above, the optimum range of growth temperatures for a particular plant species. Such optimum growth temperature ranges are readily determined or known to those skilled in the art.

15

"Dehydration stress" is any stress which is associated with or induced by the loss of water, reduced turgor or reduced water content of a cell, tissue, organ or whole plant.

"Drought stress" refers to any stress which is induced by or associated with the deprivation  
20 of water or reduced supply of water to a cell, tissue, organ or organism.

The terms "salinity-induced stress", "salt-stress" or similar term refers to any stress which is associated with or induced by a perturbation in the osmotic potential of the intracellular or extracellular environment of a cell.

25

The term "MYB2 protein" or "MYB2" or similar term shall be taken to refer to any peptide, polypeptide, monomeric or multimeric protein, fusion polypeptide, fusion protein or enzyme which comprises a sequence of amino acids which is at least about 30% identical to the amino acid sequence of *Arabidopsis thaliana* MYB2 [hereinafter referred to as "AtMYB2" (Urao  
30 *et al.*, 1993)] wherein said peptide, polypeptide, monomeric or multimeric protein, fusion

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polypeptide, fusion protein or enzyme further includes an amino acid sequence which is at least about 85%, preferably at least about 90% and more preferably at least about 95% identical to the R2 domain and/or R3 domain of AtMYB2.

5 In determining whether or not two amino acid sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments, differences will arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity or similarity between  
10 two or more amino acid sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard algorithm known to those skilled in the art. For example, amino acid sequence identities or similarities may be calculated using the GAP programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux  
15 *et al*, 1984). The GAP programme utilizes the algorithm of Needleman and Wunsch (1970) to maximise the number of identical/similar residues and to minimise the number and/or length of sequence gaps in the alignment. Alternatively or in addition, wherein more than two amino acid sequences are being compared, the ClustalW programme of Thompson *et al* (1990) is used.

20

For the purposes of clarification, the nucleotide sequence and derived amino acid sequence of AtMYB2 derived from *Arabidopsis thaliana* ecotype Columbia are set forth in <400>1 and <400>2, respectively. The nucleotide sequence and derived amino acid sequence of AtMYB2 derived from *Arabidopsis thaliana* ecotype C24 are set forth in <400>3 and  
25 <400>4, respectively.

The R2 and R3 domains of a MYB-like polypeptide are known by those skilled in the art to comprise amino acid repeat regions having 2 to 3 conserved tryptophan residues spaced approximately 18-20 residues apart in each repeat region, believed to play a critical role in  
30 stabilising the DNA-binding domain of such proteins. The particular AtMYB polypeptide

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sequence set forth in <400> 2 or <400> 4 comprises only two R2 and R3 domains, each of 51 to 53 amino acid residues in length, located at amino acid residues 20 to 72 and residues 73 to 123 thereof. The present inventors have found that whilst there is a high degree of conservation between different MYB2 proteins in these R2R3 domains, there is very little  
5 amino acid sequence identity between MYB2 proteins outside the R2/R3 domains.

The present invention clearly extends to the use of homologue, analogue or derivatives of a MYB2 protein, such as the AtMYB2 protein set forth in <400> 2 or <400> 4, the only requirement being that said homologue, analogue or derivative is capable of modulating the  
10 expression of a stress-related protein in a cell, in particular a plant cell.

In the present context, "homologues" of an amino acid sequence refer to those polypeptides, enzymes or proteins which have a similar activity in modulating expression of a stress-related gene as the amino acid sequence set forth in <400> 2 or <400> 4, notwithstanding any  
15 amino acid substitutions, additions or deletions thereto. A homologue may be isolated or derived from any plant, animal or yeast cell, tissue, organ or organism, including the plant species from which AtMYB2 is derived.

Furthermore, the amino acids of a homologous polypeptide may be replaced by other amino  
20 acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment or antigenicity, and so on.

"Analogues" encompass polypeptides having the amino acid sequence of MYB2 notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein.  
25

The term "derivative" shall be taken to refer hereinafter to mutants, parts or fragments of a complete MYB2 polypeptide as defined herein which are useful at least for the purposes of utilising the DNA-binding domain and/or transcriptional activation domain in the regulation of expression of a stress-related gene, for example in the production of fusion polypeptides.  
30 Derivatives include modified peptides in which ligands are attached to one or more of the

amino acid residues contained therein, such as carbohydrates, enzymes, proteins, polypeptides or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are particularly contemplated by the present invention. Additionally, derivatives of MYB2 which comprise fragments parts  
5 of the subject amino acid sequences are within the scope of the invention, as are homopolymers or heteropolymers comprising two or more copies of the subject polypeptides. Particularly contemplated derivatives are fusion polypeptides between MYB2 and a second regulatory polypeptide which are capable of binding to the 5'-upstream region or promoter sequence of a stress-related gene, but which are capable of modulating activity of said gene  
10 is a different manner or to a different degree compared to MYB2. For example, the extent of induction or repression of expression of the stress-related gene may be greater using such derivatives, compared to using the un-derivatised MYB2. Procedures for derivatizing proteins are well-known in the art.

15 Substitutions encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as "conservative", in which an amino acid residue contained in MYB2 is replaced with another naturally-occurring amino acid of similar character, for example Gly↔Ala, Val↔Ile↔Leu, Asp↔Glu, Lys↔Arg, Asn↔Gln or Phe↔Trp↔Tyr.

20

Substitutions encompassed by the present invention may also be "non-conservative", in which an amino acid residue which is present in MYB2 is substituted with an amino acid with different properties, such as a naturally-occurring amino acid from a different group (eg. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a  
25 naturally-occurring amino acid is substituted with a non-conventional amino acid.

Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

30 Amino acid deletions will usually be of the order of about 1-50 amino acid residues, while

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insertions may be of any length. Those skilled in the art will readily be able to determine the length of a MYB2 protein which may be deleted without significantly reducing the ability of the protein to modulate expression of a stress-related protein in a cell. Deletions and insertions may be made to the N-terminus, the C-terminus or be internal deletions or insertions.

5 Generally, insertions within the amino acid sequence will be smaller than amino- or carboxyl-terminal fusions and of the order of 1-4 amino acid residues.

Preferred homologues, analogues and derivatives of the MYB2 polypeptide described herein will comprise an amino acid sequence that is at least about 70% identical to the amino acid

10 sequence set forth in <400>2 or <400>4, and preferably at least about 80% identical, more preferably at least about 90% identical and even more preferably at least about 95% identical to said amino acid sequences. It is also particularly preferred that such homologues, analogues and derivatives are functionally-equivalent to the MYB2 polypeptides exemplified herein in so far as their ability to trans-activate the expression of a stress-related gene derived

15 from a plant species is concerned and preferably, in so far as their ability to modify the stress responses of plants, at least under conditions of anoxia, hypoxia or other anaerobic stress and/or cold stress and/or dehydration stress is concerned.

In an alternative embodiment of the invention, the subject method comprises the additional first step of introducing into the cell an isolated nucleic acid molecule which encodes a MYB2

20 protein or a homologue, analogue or derivative thereof operably connected in the sense orientation to a promoter sequence.

Optionally, the isolated nucleic acid molecule may further be operably connected to a terminator sequence.

25

The isolated nucleic acid molecule may comprise any sequence of nucleotides capable of encoding a MYB2 polypeptide or a homologue, analogue or derivative thereof as hereinbefore defined. Preferably, the isolated nucleic acid molecule is at least about 20-30% identical to

<400> 1, more preferably at least about 30-50% identical to <400> 1 and even more

30 preferably at least about 50-75% identical to <400> 1 and still more preferably at least

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about 75-95% identical to <400> 1 or a part or fragment thereof.

The isolated nucleic acid molecule may be one or more of the following molecules:

- (i) a classical genomic gene consisting of transcriptional and/or translational regulatory  
5 sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'-  
untranslated sequences);
- (ii) mRNA or cDNA corresponding to the coding region or a part thereof or one or more  
exon sequences, and 5'-untranslated sequences and/or 3'- untranslated sequences of the gene;
- (iii) a structural region corresponding to the coding region or a part thereof or one or more  
10 exon sequences; and/or
- (iv) a synthetic or fusion molecule encoding a MYB2 functional product.

Preferably, the isolated nucleic acid molecule, promoter and terminator sequences are  
contained within a genetic construct suitable for introduction into a plant cell for transient  
15 expression therein or alternatively, which is suitable for the stable transformation of the cell  
by any means known to those skilled in the art. Such genetic constructs preferably include  
one or more selectable marker genes or reporter genes for efficient selection of cells carrying  
the subject nucleic acid molecule.

20 Optionally, the genetic construct may further comprise sequences for integration into cellular  
DNA, in particular the left border (LB) and/or right border (RB) sequences of T-DNA  
required for *Agrobacterium tumefaciens* mediated transformation of plant cells.

The term "selectable marker gene" shall be taken to refer to any gene as hereinbefore defined,  
25 the expression of which in a cell may be utilised to detect and/or select for the presence of  
a transgene to which said selectable marker gene is linked or which said selectable marker  
gene has been co-transformed.

Preferred selectable marker genes include genes which when expressed are capable of  
30 conferring resistance on a cell to a compound which would, absent expression of said

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selectable marker gene, prevent or slow cell proliferation or result in cell death. Preferred selectable marker genes contemplated herein include, but are not limited to antibiotic-resistance genes such as those conferring resistance to ampicillin, Claforan, gentamycin, G-418, hygromycin, kanamycin, neomycin, spectinomycin, tetracycline or a derivative or  
5 related compound thereof or alternatively, herbicide-resistance genes such as those conferring resistance to the compounds atrazine, Basta, bialaphos, bromoxinol, Buctril, 2,4-D, glyphosate, phosphinothricin, suphonylurea or a derivative or related compound thereof, amongst others. The compound names "Basta", "Buctril", "claforan" and "G-418" are trademarks.

10

In a particularly preferred embodiment, the selectable marker gene is the neomycin phosphotransferase gene *npt II*, which when expressed confers resistance on a cell to neomycin and kanamycin and related compounds thereof. More preferably, the *nptII* selectable marker gene is placed operably under the control of a promoter suitable for  
15 expression in a plant cell.

The term "reporter gene" shall be taken to refer to any gene which, when expressed, produces a polypeptide or enzyme capable of being assayed, for example the bacterial chloramphenicol acetyltransferase gene,  $\beta$ -glucuronidase gene and firefly luciferase gene,  
20 amongst others. Those skilled in the art will be aware that the coding region of a reporter gene may be placed in operable connection with a promoter sequence such that expression of said reporter gene may be monitored to determine the pattern of expression regulated by said promoter sequence.

25 Preferred reporter genes are those genes for which their expression is capable of being assayed, for example the bacterial chloramphenicol acetyl transferase (CAT) gene, bacterial  $\beta$ -glucuronidase (*uidA*, GUS or *gusA*) gene, firefly luciferase (*luc*) gene, green fluorescent protein (*gfp*) gene or other gene which is at least useful as an indicator of expression.

30 The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which

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signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals  
5 and/or plants. Examples of terminators particularly suitable for use in the genetic constructs of the present invention include the nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays*, the ribulose -1, 5-bisphosphate carboxylase small subunit gene (*rbcS 1a* or *rbcS 3a*) terminator, and the isopentenyladenine transferase  
10 (*ipt*) terminator, amongst others.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box  
15 sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site  
20 of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of the subject *MYB2* protein in a cell, in particular a plant cell. Preferred promoters may contain additional copies  
25 of one or more specific regulatory elements, to further enhance expression of the gene and/or to alter the spatial expression and/or temporal expression. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of the *MYB2* protein, thereby conferring copper inducibility on the expression of said gene.

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Placing a gene operably under the control of a promoter sequence means positioning the said gene such that its expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in expressing a MYB2 protein in a cell include viral, fungal, animal and plant derived promoters. Preferred promoters are capable of conferring expression in a eukaryotic cell, especially a plant cell. The promoter may regulate the expression of a gene constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as environmental stress, or hormones amongst others. Examples of preferred promoters according to the present invention include, but are not limited to the CaMV 35S promoter, NOS promoter, octopine synthase (OCS) promoter, Sc4 promoter from subterranean clover stunt virus, seed-specific promoter such as the vicilin promoter or a derivative thereof, floral-specific promoter such as *apetala-3*, anther-specific promoter, tapetum-specific promoter, root-specific promoter, leaf-specific promoter such as the *Arabidopsis thaliana rbcS 1a* promoter or other *rbcS* promoter sequence, stem-specific promoter, light-inducible promoter such as the *Arabidopsis thaliana rbcS 1a* promoter or other *rbcS* promoter sequence, metal-inducible promoter such as the copper-inducible promoter, a glucocorticoid-inducible promoter, heat-shock promoter or other environmentally-inducible promoter such as those induced by anaerobiosis or hypoxia, flooding stress, cold stress, dehydration stress or salt stress, or wound-inducible promoter, amongst others. Preferred promoters may also be synthetic sequences comprising one or

- 29 -

more upstream activating sequences derived from any of the promoters referred to herein. Those skilled in the art will recognise that the choice of promoter will depend upon the nature of the cell being transformed and when expression of the recombinase, structural gene or other gene contained in the genetic construct of the invention is required.

5

In a particularly preferred embodiment, the promoter is the glucocorticoid-inducible promoter or the CaMV 35S promoter sequence and/or the terminator is the *A. thaliana rbcS 3a* terminator or the octopine synthase terminator and/or the selectable marker gene is selected from the list comprising hygromycin transferase and a gene encoding resistance to  
10 Spectinomycin, Streptomycin, ampicillin or kanamycin.

In a further preferred embodiment of the invention, the isolated nucleic acid molecule encoding MYB2 is contained within a genetic construct diagrammatically represented in the accompanying Figures.

15

Although intended for the transformation of a eukaryotic organism and/or the expression of genes contained therein, the genetic constructs of the present invention may need to be propagated in a prokaryotic organism such as the bacteria *Escherichia coli* or *Agrobacterium tumefaciens*. Accordingly, the genetic constructs described herein may further comprise  
20 genetic sequences corresponding to a bacterial origin of replication and/or a selectable marker gene such as an antibiotic-resistance gene, suitable for the maintenance and replication of said genetic construct in a prokaryotic organism. Such sequences are well-known in the art. Usually, an origin of replication or a selectable marker gene suitable for use in bacteria is physically-separated from those genetic sequences contained in the genetic construct which  
25 are intended to be expressed or transferred to a eukaryotic cell, or integrated into the genome of a eukaryotic cell.

Those skilled in the art will be aware of how to produce a genetic construct expression of the MYB2 protein and of the requirements for obtaining the expression thereof, when so desired,  
30 in a specific cell or cell-type under the conditions desired.

- 30 -

The present invention extends to all genetic constructs essentially as described herein.

The genetic constructs described herein are particularly useful for the production of stress-tolerant transgenic cells and transgenic organisms, in particular transgenic plants.

5

Accordingly, a further aspect of the invention provides a stress-tolerant cell or organism and methods of producing same.

The term "transgenic organism" refers to any organism that has a nucleic acid molecule, including, but not limited to DNA, cDNA, mRNA, tRNA, rRNA, synthetic oligonucleotide molecule, ribozyme, antisense molecule, co-suppression molecule, structural gene, wherein said nucleic acid molecule is introduced into the genome of a cell as an addition to the complement of genetic material present in said cell in the absence of said nucleic acid molecule.

15

By "stress-tolerant transgenic" is meant that the transgenic cell or organism is able to withstand a particular stress for a longer period of time than an otherwise isogenic non-transgenic organism without resulting in cell damage or death.

20 The term "stress-tolerant transgenic" also encompasses transgenic cells and organisms in which a stress response occurs more rapidly or to a greater degree, for example by inducing expression of an ANP compared to an otherwise isogenic non-transgenic cell or organism. The earlier or more intense expression of a stress-related protein, in particular an ANP, enables a cell to modify its metabolism as part of an adaptive response earlier than would otherwise be the case. However, the enhanced survival of the cell is probably also dependent upon the repression of expression of stress-related proteins which are not ANPs, because the amino acids normally required for the synthesis of such proteins in the absence of stress, must be diverted into synthesis of ANPs. Accordingly, the present invention also encompasses stress-tolerant plants wherein one or more genes encoding a non-ANP stress-related protein  
30 is repressed by a MYB2 protein.

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Preferably, the stress-tolerant cell or organism is at least tolerant to anaerobic stress and/or flooding stress and/or cold stress. Compared to otherwise isogenic non-transformed cells and organisms, the stress-tolerant cell or organism preferably has increased expression levels of alcohol dehydrogenase and/or pyruvate decarboxylase enzymes in the absence of stress and/or immediately following stress induction and/or is capable of inducing expression of these enzymes earlier.

Methods of producing a transgenic plant are well-known to those skilled in the art. The technique used for a given plant species or specific type of plant tissue depends on the known successful techniques.

Means for introducing recombinant DNA into plant tissue include, but are not limited to, direct DNA uptake into protoplasts (Krens *et al.*, 1982; Paszkowski *et al.*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al.*, 1990) microparticle bombardment electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al.*, 1988; Sanford, 1988), vacuum-infiltration of plant tissue with nucleic acid, or T-DNA-mediated transfer from *Agrobacterium* to the plant tissue. Representative T-DNA vector systems are described in the following references: An *et al.* (1985); Herrera-Estrella *et al.* (1983a,b); Herrera-Estrella *et al.* (1985).

For microparticle bombardment of cells, a microparticle is propelled into a plant cell, in particular a plant cell not amenable to *Agrobacterium* mediated transformation, to produce a transformed cell. Wherein the cell is a plant cell, a whole plant may be regenerated from the transformed plant cell. Alternatively, other non-animal cells derived from multicellular species may be regenerated into whole organisms by means known to those skilled in the art. Any suitable ballistic cell transformation methodology and apparatus can be used in practising the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid

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capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5  $\mu\text{m}$  gold spheres.

The DNA construct may be deposited on the microparticle by any suitable technique, such  
5 as by precipitation.

Plant species may be transformed with the genetic construct of the present invention by the  
DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the  
plant from the transformed protoplasts in accordance with procedures well known in the art.

10

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or  
embryogenesis, may be transformed with a vector of the present invention. The particular  
tissue chosen will vary depending on the clonal propagation systems available for, and best  
suited to, the particular species being transformed. Exemplary tissue targets include leaf  
15 disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing  
meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced  
meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are  
20 developed sequentially from meristematic centers.

The term "embryogenesis", as used herein, means a process by which shoots and roots  
develop together in a concerted fashion (not sequentially), whether from somatic cells or  
gametes.

25

Plants of the present invention may take a variety of forms. The plants may be chimeras of  
transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all  
cells transformed to contain the MYB2 expression cassette); the plants may comprise grafts  
of transformed and untransformed tissues (e.g., a transformed root stock grafted to an  
30 untransformed scion in citrus species). The transformed plants may be propagated by a

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variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques.

5

Plants which may be employed in practising the present invention include both horticultural plant species and agricultural plant species, tree species and ornamental plant species.

Preferred recipient plants include *Arabidopsis thaliana*, wheat, barley, rice, rye, maize, or  
10 sorghum, oil seed rape (*Canola*), *Linola*, cotton and sugar cane. Additional species are not excluded.

Once introduced into the plant tissue, the expression of the introduced gene may be assayed in a transient expression system, or it may be determined after selection for stable integration  
15 within the plant genome. Techniques are known for the *in vitro* culture of plant tissue, and in a number of cases, for regeneration into whole plants. Procedures for transferring the introduced genetic construct from the originally transformed plant into commercially useful cultivars are known to those skilled in the art.

20 The present invention further extends to the progeny of said transgenic plant.

The present invention is further described with reference to the accompanying non-limiting Figures and Examples.

25

#### EXAMPLE 1

##### Plant material, growth conditions and stress treatments.

*Arabidopsis thaliana* seeds, ecotypes C24 or Columbia (Co-O), used in this study were grown on Murashige and Skoog (MS) medium at 22°C (16/8 hours light/dark cycle, 200  $\mu$ E.s-1.cm-2). Stress and ABA treatments were carried out hydroponically, in dishes  
30 containing 15 ml liquid MS medium as previously described (Dolferus *et al.*, 1994; de

Bruxelles *et al.*, 1996). Low oxygen treatments were carried out by incubating plantlets in a 5% O<sub>2</sub>/95% N<sub>2</sub> gas mixture (hypoxic conditions; Howard *et al.*, 1987), for 24 h at 220 C in the dark. Dehydration treatment was carried out by incubating the plantlets in medium containing 0.6 M mannitol, for 24 h at 220 C. For cold stress treatment, plantlets were  
5 incubated at 4-50 C for 24 h. Absciscic acid (ABA, (±) cis-trans isomers, Sigma) was added to the medium at a final concentration of 0.1 mM for 4 h. For treatments with the protein synthesis inhibitor cycloheximide, plant material was first pre-incubated in MS medium containing 10 µM cycloheximide for 1 h. The solution containing cycloheximide was refreshed for incubation during the stress treatment.

10

## EXAMPLE 2

### Isolation of a full-length AtMYB2 cDNA

The nucleotide sequence of the *Arabidopsis thaliana* ecotype Columbia AtMYB gene is presented in <400>1, with the corresponding amino acid sequence encoded therefor  
15 presented in <400>2.

A full-length AtMYB2 cDNA, flanked by *Bam*HI sites was obtained using RT-PCR of anaerobically induced root RNA derived from *Arabidopsis thaliana* ecotype C24. The 5'-end and the 3'- end of the cDNA were each amplified and subcloned separately and their identities  
20 confirmed by nucleotide sequence analysis. A *Bam*HI site was created at both the 5'-end and 3'- end of the cDNA and an *Xho*I site within the cDNA was used to amplify both ends as *Bam*HI/*Xho*I fragments. The resultant plasmids were called pCMYB/5'(5'-end; Figure 9) and pCMYB/3' (3'-end; Figure 10). These clones were used to reconstruct the complete cDNA as a *Bam*HI fragment in plasmid pGEM3Zf(-), producing the plasmid pCATMYB2/S; Figure  
25 11).

The nucleotide sequence of the full-length cDNA present in pCATMYB2/S is set forth in <400>3. The nucleotide sequence of the cDNA differs in a few positions from the genomic sequence derived from ecotype Columbia (GenBank Accession No. D14712).

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### EXAMPLE 3

#### Gel Retardation assays

All cloning methods were according to standard procedures (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989). Plasmid pGEX-RAtmyb 2BE, containing the AtMYB2 cDNA fused to the glutathione-S-transferase coding region in plasmid pGEX2T was kindly supplied by Dr. Shinozaki (Urao *et al.*, 1993). The AtMYB2 fusion protein was purified using the Pharmacia GST purification module and used in GMSA experiments. Complementary oligonucleotide probes used in gel retardation assays (see Figure 1-2 ) were annealed, end-labeled using Klenow DNA polymerase, and then gel-purified. Binding reactions contained 1  $\mu$ l recombinant AtMYB2 (1:100 diluted; about 50 ng protein), 2  $\mu$ l 10x EMSA buffer (100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM EDTA, 0.5% skimmed milk powder, 50% glycerol, and 10 mM DTT), 1  $\mu$ l DTT (10 mM), 1  $\mu$ l poly dI-dC (1  $\mu$ g/ $\mu$ l), 15  $\mu$ l H<sub>2</sub>O, and 1  $\mu$ l labelled target oligonucleotide (0.1 ng; 10,000 cpm). Reactions were incubated at room temperature for 10-15 min. Competition experiments were performed by adding unlabeled competitor oligonucleotide to the reaction prior to the addition of radiolabeled oligonucleotide. Samples were loaded on a 5% polyacrylamide gel in 0.5x TBE (Maniatis *et al.*, 1982).

20

### EXAMPLE 4

#### RNA extractions, northern and southern blot analysis

RNA extraction, gel electrophoresis, northern blot hybridisations using antisense RNA probes, and filter washing procedures were completely as described previously (Dolferus *et al.*, 1994). Filters were placed on phosphor imager screens (Molecular Dynamics, Sunnyvale, CA) and the hybridization signals quantified. Uniformity of gel-loading was assessed visually using ethidium bromide staining. The Arabidopsis ubiquitin gene (Burke *et al.*, 1988) was used as a probe to correct for variation in sample loading, by dividing all signal strengths by their respective ubiquitin signal. Quantitative RT-PCR was carried out using 1  $\mu$ g total RNA and the Promega Access RT-PCR system (Madison, WI). Samples were taken during the

30

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PCR reaction after 5, 10, 15 and 25 cycles and loaded on agarose gels. Gels were treated for Southern blot hybridisation, and filters were hybridised using the AtMYB2 cDNA. Linearity of signal strength was verified using phosphorimager quantifications. Oligonucleotides were used slightly overlapping the 5' end and 3' end of the first and second intron positions of the AtMYB2 gene respectively. These oligonucleotides were shown not to amplify using genomic DNA as template.

### EXAMPLE 5

#### Tissue culture, protoplast transient assays, particle bombardment and *Agrobacterium*-mediated transformation.

10

Binary vectors were mobilised to *Agrobacterium* strain AGL1 (Lazo *et al.*, 1991) by electroporation (Nagel *et al.*, 1990).

*Arabidopsis* root cultures were established by placing one-month-old leaf cuttings (ecotype C24) on callus-induction medium (CIM; Valvekens *et al.*, 1988) for three days, prior to infection with *Agrobacterium rhizogenes* (strain A4RS; Vilaine and Casse-Delbart, 1987). The leaf disks were co-cultivated for three days on CIM-medium, washed in a 200 mg/ml timentin solution (Beecham), and placed on solid MS-medium including 100 mg/ml timentin. After 3-4 weeks the explants were transferred to liquid MS medium, and refreshed monthly.

Transgenic *Arabidopsis* plants were obtained using a slightly modified root explant transformation method (Valvekens *et al.*, 1988; Dolferus *et al.*, 1994). *Arabidopsis* mesophyll protoplasts were prepared from ecotype Co-O, using a modification of previously published procedures (Damm and Willmitzer, 1988; Damm *et al.*, 1989; Abel and Theologis, 1994). Typically, transient assays were carried out using  $2.10^{-6}$  protoplasts and 15-20  $\mu$ g reporter plasmid DNA, plus or minus the same amount of effector plasmid (p35S-cAtMYB2/S; Figure 12). Suspension cell protoplasts from *N. plumbaginifolia* were prepared according to Negrutiu *et al.* (1981), and plasmid DNA was introduced using the same methods as for *Arabidopsis* protoplasts.

Particle bombardment of pea leaves was carried out using a home made helium gun. A mixture

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of tungsten and gold particles (50:50 ratio) was used as coating material: 25  $\mu$ l of the particles (100mg/ml in 50% glycerol) were mixed with 2-6  $\mu$ g plasmid DNA (1  $\mu$ g/  $\mu$ l), 25  $\mu$ l 2.5 M CaCl<sub>2</sub>, and 10  $\mu$ l spermidine (0.1 M). The total volume was then adjusted to 40  $\mu$ l, and 4  $\mu$ l was used for the bombardment of 1 leaf. Reporter and effector plasmid were used in a 1:1 ratio. Leafs were incubated on MS plates for 16 h before GUS staining.

### EXAMPLE 6

#### GUS histochemical staining and fluorometric assays

GUS fluorometric assays and in vivo histochemical stainings were carried out as previously described (Jefferson, 1987; De Block and De Brouwer, 1992). The total protein concentration in the extracts was determined using a Bio-Rad protein assay kit (Bradford, 1976). Fluorometric and protein assays were carried out in microwell plates, and analysed using Labsystems Fluoroskan II and Multiskan Plus readers, in conjunction with Delta Soft II software (Biometallics Inc., Princeton, USA; Breyne *et al.*, 1993). For in vivo GUS assays, the staining solution was vacuum infiltrated into the plant tissue. Plant material was fixed in 70% ethanol after GUS staining.

### EXAMPLE 7

20           **The *Arabidopsis ADH1* promoter has binding sites for AtMYB2.**

Nucleotide sequences of oligonucleotides containing the wild-type *Adh1* promoter sequence and several mutant sequences were derived from the sequences of known genetic constructs (Dolferus *et al.*, 1994). The nucleotide sequences of the wild-type sequence and these mutations are set forth in Figures 1-1 and 1-2 herein.

25

The MBS-2 site was mutated by amplifying fragments overlapping the MBS2 site (5'-TAGCAACGCC-3') and transforming this site into a *NotI* restriction site (5'-GCGGCCGCAT-3'). The nucleotide sequences of the wild-type sequence and these mutations contained therein are set forth in Figures 1-1 and 1-2 herein.

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The AtMYB2 gene was expressed in *E. coli* as a glutathione-S-transferase fusion protein and affinity purified. This fusion protein was used in electrophoretic mobility shift assays (EMSA), to assay binding to oligonucleotides corresponding to the MBS-1 (GT-motif) and MBS-2 regions of the *ADHI* promoter (Figure 1-2). The recombinant AtMYB2 protein  
5 retarded oligonucleotides containing both MBS-1 (GT-motif) and MBS-2 binding sites (Figure 2-1). Binding was marginally stronger for the oligonucleotides containing the GT-motif. No binding was observed for oligonucleotides containing either the G-box-1 or GC-motif sequences, nor did the recombinant *Arabidopsis* AtMYB2 protein (Urao *et al.*, 1993) bind to the MBS-1 and MBS-2 oligonucleotides (data not shown). Multimerisation of the MBS-1  
10 (GT-motif) oligonucleotide gave significantly stronger binding than the monomers (Figure 2-2). Binding to the monomers was competed out by hundred to two hundred-fold molar excess for the MBS-1 and MBS-2 oligonucleotides. Higher amounts of cold multimeric oligonucleotide were required to compete out binding to the tetramers, with 500-fold molar excess excluding all binding (Figure 2-2). Addition of non-specific competitors such as the  
15 G-box-1 and GC-motif sequences did not compete for binding (Figure 2-1).

### EXAMPLE 8

#### AtMYB2 binding to MBS-1 and MBS-2 requires the AAC-core.

We tested the specificity of binding of AtMYB2 by determining whether the central  
20 AAC-core sequence in the MBS-1 and MBS-2 sequences was essential (Figure 2-3). Mutations were introduced within the core sequence (Figure 1-2), and the effect on binding to AtMYB2 was determined using EMSA (Figure 2-3). The first mutation tested was a substitution, C for A, in the first A of the AAC-core in the MBS-1 and MBS-2 motifs. A similar mutation in a vertebrate system (Weston *et al.*, 1992) abolished Myb-binding, and the  
25 same result was seen in the case of the plant GA-Myb (Gubler *et al.*, 1995). This substitution did not abolish binding of AtMYB2 to either motif, but greatly reduced binding to each of the substituted boxes relative to the wild type sequence (data not shown). Replacing the AAC core with CCC (MBS1/1) completely abolished binding to both sites, and the mutated oligonucleotides were unable to compete out binding by the wild type sequence (Figure 2-3).  
30 When the AAC core sequence was mutated to GAC (MBS-1/2), some degree of competition

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with wild type MBS-1 binding remained (Figure 2-3). The MBS-1 (5'-AAACCAAA-3') and MBS-2 (5'-CAACGCCA-3') sequences competed with each other despite differences in sequence (data not shown). In conclusion, the EMSA results indicate that the potential Myb binding sites in the *ADHI* promoter, MBS-1 (GT-motif) and MBS-2 (upstream of the G-box-2  
5 sequence), are each able to interact with AtMYB2, and that the AAC sequence is an important core element for both these interactions.

### EXAMPLE 9

#### AtMYB2 expression is induced by low oxygen stress in roots.

10 We investigated the effect of low oxygen stress on AtMYB2 expression in roots and leaves of one-month-old *Arabidopsis* plantlets (Figure 3-1). Maximal induction levels for *ADHI* are obtained after 4-6 hours of low oxygen stress treatment (Dolferus *et al.*, 1994; de Bruxelles *et al.*, 1996). Low oxygen inductions were carried out for 4 hours. We found that AtMYB2 is induced strongly by low oxygen (average: 5.6-fold induction), and expression is  
15 significantly higher in roots than in shoots (about 7 times on average; Figure 3-1 and Figure 3-2).

The timing of AtMYB2 induction by low oxygen stress was compared to that of *ADHI*, using RNA extracted from *Arabidopsis* root cultures (Figure 3-3). AtMYB2 levels peak after 2-4  
20 hours, preceding maximal levels of *ADHI* mRNA (4-6 hours). The induction of AtMYB2 is also coordinated with the induction of other anaerobically-induced *Arabidopsis* genes, such as the pyruvate decarboxylase (PDC1; Dolferus *et al.*, in preparation), and sucrose synthase genes (*ASUSI*; Martin *et al.*, 1993; Figure 4). All these genes contain GT-motifs which are potential binding sites for AtMYB2 (Table I). These genes show similar induction kinetics  
25 following low oxygen treatment, with peak expression levels found immediately after AtMYB2 mRNA levels have reached a maximum, suggesting that AtMYB2 is required for their activation following low oxygen stress.

**EXAMPLE 10**

**AtMYB2 is able to transactivate *ADHI*.**

**1. Base Plasmids****A. The effector plasmid p35S-CAtMYB2/S**

- 5 The full-length AtMYB2 cDNA was sub-cloned from plasmid pCATMYB2/S (Figure 11) into plasmid pART7 (Gleave, 1992), between the 35S promoter and the 3'*NOS* terminator sequence. The resulting plasmid, designated p35S-CAtMYB2/S (Figure 12) was verified by sequencing and used as effector plasmid in all transient assays.

**10 B. The reporter plasmid pCADH-GUS**

The reporter plasmid pCADH-GUS is described previously (Dolferus et al., 1994). The map of this plasmid is set forth in Figure 13.

**2. Experimental**

- 15 To investigate whether AtMYB2 could transactivate hypoxically induced genes in the absence of hypoxia, we set up two different transient assay systems. As reporter construct we used an *ADHI*-promoter-GUS fusion containing all *cis* elements of the *ADHI* promoter necessary for anaerobic induction (CADH-GUS; Figure 13; Dolferus *et al.*, 1994). The effector plasmid was a 35S Cauliflower mosaic virus promoter, driving a full-length AtMYB2 cDNA  
20 (p35S-CAtMYB2/S; Figure 12). Pea leaves were used as target tissue and DNA was introduced by biolistics. The AtMYB2 gene product did activate the ADH-GUS reporter plasmid as shown by the overall increased intensity of GUS staining and the size of the blue spots on the leaves co-bombarded with pCADH-GUS and p35S-AtMYB2/S. Data are presented in Figure 5. This experiment was repeated four times in duplicate and gave  
25 essentially similar results, with a 2-3 fold enhancement of *ADHI*-promoter-driven GUS expression in the presence of the 35S-AtMYB2 construct.

In order to obtain more quantitative data about AtMYB2 transactivation of the *ADHI* promoter, we carried out transient assays using protoplasts with the same constructs.

- 30 AtMYB2 expression was able to transactivate *ADHI* promoter activity by a factor of 2-2.5

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(Figure 6-1). Higher stimulations of ADH1-promoter-driven GUS expression were found when *Nicotiana plumbaginifolia* suspension cell protoplasts were used (Figure 6-1; about 2.5-4.5-fold stimulation on average). Levels of transactivation were not increased by higher amounts of effector plasmid compared to the reporter plasmid (data not shown).

5

### EXAMPLE 11

**AtMYB2 transactivates the *ADH1* promoter via the GT-motif (MBS-1).**

#### 1. Base Plasmids

##### A. The effector plasmid p35S-CAtMYB2/S

10 Plasmid p35S-CAtMYB2/S (Figure 12) is described in the preceding Example.

##### B. The reporter plasmid pCADH-GUS and derivatives thereof

The reporter plasmid pCADH-GUS is in the preceding Example. Plasmids p35S-GUS, p[GBox-1]ADH-GUS, p[GBox-2]ADH-GUS p[GT]ADH-GUS p[GC]ADH-GUS were  
15 described earlier (Dolferus *et al.*, 1994).

Plasmid p[MBS-2]ADH-GUS, containing a deletion in the MBS-2 site (Figures 1-1 and 1-2), was constructed by amplifying fragments overlapping the MBS2 site (5'-TAGCAACGCC-3') and transforming this site into a *NotI* restriction site (5'-GCGGCCGCAT-3').

20

#### 2. Experimental

The nucleotide sequences of the wild-type *Adh1* sequence and the mutations contained therein are set forth in Figures 1-1 and 1-2 herein.

25 Mutations in the GT-motif decreased ADH1 expression drastically (Dolferus *et al.*, 1994).

The presence of a second potential Myb binding site MBS-2 in the *ADH1* promoter which is able to bind AtMYB2 suggested the *Arabidopsis ADH1* promoter may resemble the maize *Adh1* promoter more closely, where two GT-motifs were shown to be functionally important (Figure 1-1). MBS-2 is located in a region which is part of a larger in vivo footprinted area.

30 The area previously mutagenised within the G-box-2, did not affect *ADH1* expression

(Dolferus *et al.*, 1994).

We mutagenised the MBS-2 region (5'-TAGCAACGCC-3') to a NotI restriction site (5'-GCGGCCGCAT-3'; p( $\Delta$ AMBS-2)CADH-GUS) and checked the effect of these changes  
5 on GUS expression and transactivation by AtMYB2. Using *Arabidopsis* mesophyll protoplasts, we found that AtMYB2 was unable to transactivate constructs with a mutated MBS-1 (GT-motif; Dolferus *et al.*, 1994) sequence, but the transactivation potential of AtMYB2 was not affected by mutations in the MBS-2 sequence (Figure 6-2). Essentially the same results were obtained using *N. plumbaginifolia* protoplasts. These data indicate that,  
10 although AtMYB2 is able to bind to the MBS-2 site, binding is not functional and MBS-2 is, in the presence of an intact MBS-1 site, not of prime importance for the *ADH1* promoter.

Mutation of the GC-motif (Figure 1-2; Dolferus *et al.*, 1994) strongly reduced AtMYB2 transactivation of ADH-GUS expression in both *Arabidopsis* mesophyll protoplasts and in  
15 *N. plumbaginifolia* suspension cell protoplasts (Figure 6-2). These results indicate that anaerobic induction of the *ADH1* promoter requires AtMYB2 and the GT-motif, but also a factor binding to an intact GC-motif plays an important role in promoter activation. Transactivation levels of the G-Box-1 mutants (Figure 1-2) were also reduced compared to the wild type promoter in both *Arabidopsis* mesophyll and *N. plumbaginifolia* suspension  
20 protoplasts, with transactivation levels similar to the GC-mutants (Figure 6-2), indicating that the G-box binding factor plays a role in stabilising binding of AtMYB2 to the promoter. Close to normal transactivation levels were found for the G-Box-2 mutants (Figure 1-2) in both *Arabidopsis* and *N. plumbaginifolia* protoplasts, suggesting that this mutation is not interfering dramatically with the assembly of a functional promoter complex. This result is  
25 consistent with the results obtained in transgenic plants, where the effect of G-Box-2 mutants was less obvious than G-Box-1 mutants (Dolferus *et al.*, 1994; de Bruxelles *et al.*, 1996).

#### EXAMPLE 12

**Induction of *ADH1* by stress requires protein synthesis but AtMYB2 is hyper-induced.**  
30 Protein synthesis is required before ADH1 mRNA is induced by low oxygen, dehydration,

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ABA, and low temperature conditions (Figure 7-1). The addition of cycloheximide (10  $\mu$ M) 1 h prior to and during stress treatment prevents accumulation of *ADHI* mRNA. Because AtMYB2 accumulation is induced by the same stress treatments, we tested the effect of cycloheximide on AtMYB2 expression using a combination of linear RT-PCR and Southern blot hybridisation (Figure 7-2). The results indicate that cycloheximide treatment caused a massive increase in AtMYB2 mRNA levels for all treatments (100-800%). It is not clear at this stage whether this effect of cycloheximide is at the transcriptional or post-transcriptional level, or due to increased mRNA stability. Experiments with AtMYB2-promoter-GUS constructs have indicated that stress-induced expression of AtMYB2 is at least partially controlled at the transcriptional level (Urao *et al.*, 1993). This indicates that AtMYB2 can be induced without protein synthesis occurring, while *ADHI* induction does depend on protein synthesis.

### EXAMPLE 13

#### 15 Induction of AtMYB2 by other stresses correlates with *ADHI* induction.

Maximal induction levels for *ADHI* are obtained after 8-10 hours of dehydration stress, 20-24 hours of low temperature, and 4 hours of ABA treatment (Dolferus *et al.*, 1994; de Bruxelles *et al.*, 1996). AtMYB2 is induced by all these treatments, including low temperature which was not previously shown to induce AtMYB2; (Figure 3-1). Expression is significantly higher in roots than in shoots for low temperature treatment, but expression levels are similar in leaves and roots for dehydration and ABA treatment, even though *ADHI* is expressed predominantly in roots in response to these treatments (Figure 3-1). Equal induction levels of AtMYB2 in leaves and roots for both dehydration and ABA treatments agrees well with the fact that dehydration treatment is associated with increased ABA levels in Arabidopsis leaves and roots (de Bruxelles *et al.*, 1996). The root-specificity of *ADHI* expression under these conditions stresses the involvement of other factors, eg. the factor binding to the G-box-1 sequence.

The kinetics of AtMYB2 mRNA accumulation correspond well with the induction kinetics of *ADHI* mRNA. Two peaks (4 and 10 hours) were obtained for dehydration, which is in

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agreement with the two phases in the time course of *ADH1* mRNA induction (Figure 3-3). Low temperature treatment resulted in transient AtMYB2 mRNA accumulation between 2 and 6 hours (5-fold induction), followed by induction of *ADH1* mRNA reaching peak levels between 12 and 24 hours. We also found two peaks for ABA induction (2 and 10 hours; 24- and 15-fold induction respectively), which was mimicked by the *ADH1* time course (peaks at 4 and 10 hours; Figure 3-3). AtMYB2 expression is correlated well both temporally and spatially with expression of the *ADH1* gene: both genes are induced in a coordinated way by the same stresses and both show root-specific expression.

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#### EXAMPLE 14

##### Effects of over-expressing AtMYB and derivatives thereof in transgenic plants

Truncated versions of the *AtMYB2* cDNA which lacked the region of the gene encoding the C-terminal protein of the ATMYB protein were obtained by PCR, using the following primers:

- 15 5' Primer: 5'-GGATCCGAAATGGAAGATTACGAGCG-3'  
3' Primer ( $\Delta 251$ ): 5'-GGATCCTTACCACATGTTGTCCCCACCAACACAGC-3'  
3' Primer ( $\Delta 207$ ): 5'-GGATCCTTAAGAATTCGAAGACGTTGCTGAC-3'

In all cases, the 5' Primer contained the translation start site of the gene.

20

One truncated gene fragment was produced using the 5' Primer in combination with the 3' Primer ( $\Delta 251$ ) and is translated to yield a polypeptide product that is truncated at amino acid position 251 of AtMYB. The fragment was sub-cloned as a *Bam*HI fragment into pGEM3Zf(-) to produce the clone designated pCATMYB2 ( $\Delta 251$ ) (Figure 14).

25

Another truncated gene fragment was produced using the 5' Primer in combination with the 3' Primer ( $\Delta 207$ ) and is translated to yield a polypeptide product that is truncated at amino acid position 207 of AtMYB. The fragment was sub-cloned as a *Bam*HI fragment into pGEM3Zf(-) to produce the clone designated pCATMYB2 ( $\Delta 207$ ) (Figure 15).

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The truncated cDNAs in plasmids pCATMYB2 (251) and pCATMYB2 (207) were used to make constructs for expression studies in transgenic *Arabidopsis thaliana* plants. In a first attempt, we used the strong constitutive CaMV 35S promoter to drive expression of the truncated AtMYB2 cDNAs. The cDNAs were cloned as *Bam*HI fragments into plasmid  
5 pART7 (Gleave, 1992), producing plasmids p35S-CATMYB2(251) (Figure 16) and p35S-CATMYB2(207) (Figure 17), respectively.

The chimeric genes containing the full-length AtMYB coding region (p35S-cATMYB2/S; Figure 12) and the truncated versions p35S-CATMYB2(251) (Figure 16) and p35S-  
10 CATMYB2(207) (Figure 17) were transferred as *Not*I fragments into the binary vector pART27 (Gleave, 1992) for transformation using *Agrobacterium tumefaciens*. The resultant plasmids, pTi35S-cAtMYB2/S, pTi35S-cAtMYB2/S(251) and pTi35S-cAtMYB2/S(207) are represented in Figure 18.

15 Transgenic *Arabidopsis thaliana* plants have been obtained for all these constructs. Plants containing the plasmid pTi35S-cAtMYB2/S were analysed by northern blot hybridisation. None of the 14 independent transformants showed over expression of AtMYB2 mRNA. Whilst not being bound by any theory or mode of action, these data suggest that constitutive over-expression of AtMYB2 in *Arabidopsis thaliana* may be lethal, and possibly leads to  
20 selection of transformants where the gene is deleted or inactivated. This possibility is investigated using Southern blot hybridisations.

To avoid a possible lethal effect of AtMYB2 expression in transgenic plants, we prepared a set of new constructs with AtMYB2 under the control of a glucocorticoid-inducible promoter  
25 (Figures 19-1, 19-2, 19-3, 19-4 and 19-5). The full-length and truncated versions of the AtMYB2 cDNAs were subcloned into plasmid pTA7002 (Aoyama and Chua, 1997) in the sense orientation. Plasmid pTA7002 allows glucocorticoid-mediated transcriptional induction in transgenic plants. The cDNAs were isolated as *Xho*I(partial)/*Xba*I fragments from plasmids p35S-cAtMYB2/S (Figure 12), p35S-CATMYB2(251) (Figure 16) and p35S-cAtMYB2(207)  
30 (Figure 17), and cloned into the *Xho*I/*Spe*I sites of plasmid pTA7002, to yield plasmids pTA-

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CATMYB2 (Figure 19-1), pTA-CATMYB2(251) (Figure 19-2) and pTA-CATMYB2(207) (Figure 19-3). These constructs were transformed to *Arabidopsis thaliana*.

Transgenic plants are analysed for improved stress tolerance.

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#### EXAMPLE 15

##### Effects of under-expressing AtMYB and derivatives thereof in transgenic plants

In order to study the effect of decreased levels of *AtMYB2* gene expression on *ADH1* expression, we created antisense constructs. The full-length *AtMYB2* cDNA was subcloned  
10 as a *Bam*HI fragment into plasmid pART7 to yield plasmid p35S-cAtmyb2/A (Figure 20). The presence of the *AtMYB2* gene in the antisense orientation in this plasmid was confirmed by sequencing.

The chimeric construct was then subcloned as a *Not*I fragment in plasmid pART27, to give  
15 pTi35S-cAtmyb2/A (Figure 21). This construct was transformed to *Arabidopsis thaliana* and the resulting transgenic plants were analysed by northern blot hybridisations. A total of 12 independent antisense transformants were analysed and preliminary data show that there is no significant effect of CaMV 35S promoter-driven expression of the antisense *AtMYB2* gene on the expression of the endogenous *AtMYB2* gene, nor is there any apparent effect on *ADH1*  
20 gene expression.

In order to anticipate any problems with lethality of constitutive *AtMYB2* under expression in transgenic *Arabidopsis thaliana* plants, we constructed an antisense construct using the glucocorticoid-inducible promoter. To construct plasmid pTA-CATMYB2/A (Figure 22), we  
25 isolated the *AtMYB2* cDNA as a *Sal*II/*Sma*I fragment from pCATMYB2/S (Figure 11) and cloned this fragment into the *Xho*I/*Spe*I (filled-in) sites of pTA7002.

Different Myb proteins show a higher degree of sequence similarity in the DNA binding domain, located at the amino terminal end of the protein. In contrast, the carboxyl terminal  
30 end of the protein contains the transactivation domain of the protein, and shows a high degree

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of variability. As a consequence, we made a further antisense construct with only the —COOH end of the AtMYB2 protein, in order to avoid lethal effects caused by non-specific suppression of other Myb genes due to their homology with AtMYB2 in the DNA binding domain. The —COOH-encoding part of the AtMYB2 cDNA was obtained as a *Bam*HI PCR 5 fragment using the following primers.

5' Primer( $\Delta$ NH<sub>2</sub>): 5'-GGATCCGACTATGAGAAATGTTTGGATGCCGAG-3'

3' Primer: 5'-GGATCCTTAATTATACGAATACGATGTCGTATCG-3'

The amplified DNA was cloned into the *Bam*HI site of plasmid pQE30, to produce the 10 plasmid designated pQE $\Delta$  (NH<sub>2</sub>)AtMYB2 (Figure 23). The sequence of the amplified fragment was confirmed and the 5'-truncated gene was subsequently subcloned as a *Bam*HI fragment into the kanamycin-resistant version of pBluescript (pKBS) (Stratagene), to give plasmid pKBS $\Delta$ (NH<sub>2</sub>)AtMYB2 #29 (Figure 24). The  $\Delta$ (NH<sub>2</sub>)AtMYB2 gene fragment from this plasmid was then cloned as an *Xho*I/*Spe*I fragment into the corresponding sites of 15 pTA7002 to give plasmid pTAA(NH<sub>2</sub>)ATMYB2/A (Figure 25).

The antisense plasmids pTAA $\Delta$ CATMYB2/A (Figure 22) and pTAA $\Delta$ (NH<sub>2</sub>)ATMYB2/A (Figure 25) have been confirmed by sequence analysis. The plasmids are used to transform *Arabidopsis thaliana* to modify the stress responses of plants.

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## EXAMPLE 16

### DISCUSSION

Several lines of evidence support the idea that the transcription factor AtMYB2 is a key 25 regulatory factor in environmentally-induced *ADH1* gene expression. First, AtMYB2 binds to two potential Myb binding sites in the *Arabidopsis ADH1* promoter, the GT- (MBS- 1 ) and (MBS-2) motifs. The presence of two Myb binding sites in the *ADH1* promoter, both resembling the GT-motifs found in the maize *Adh1* promoter, suggested that *Arabidopsis ADH1* may have a double GT-element like the maize ARE (Figure 1-1). The spacing between 30 the two GT elements is different in *Arabidopsis* and maize, but variations in spacing between

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GT-motifs do not affect performance of the maize ARE (Olive *et al.*, 1991a, b). Mutations in the GT-motif (MBS-1) which eliminated binding of AtMYB2, also eliminated *ADH1* expression, both uninduced and following treatment with low oxygen tension, dehydration, low temperature or ABA (Dolferus *et al.*, 1994; de Bruxelles *et al.*, 1996). MBS-2 is located 5 just upstream of the previously mutated G-Box-2 sequence, which is not critical for expression of *ADH1* (Dolferus *et al.*, 1994). Because the previous analysis concentrated on residues resembling a G-Box motif, the mutations introduced did not remove key residues from the MBS-2 site (Dolferus *et al.*, 1994; see Figure 1-1). Transient expression assays using constructs mutated in the MBS-2 site showed that this AtMYB2 binding site is not 10 important for *ADH1* promoter activity (Figure 6-2), and that AtMYB2 binding to MBS-2 on its own is not functionally important. The GT-motif (MBS-1) is crucial for activation of gene expression by AtMYB2.

Secondly, AtMYB2 has an expression pattern that is similar to *ADH1*, and AtMYB2 mRNA 15 accumulation is tightly coupled to *ADH1* expression. Highest levels of expression of AtMYB2 mRNA precede peak levels of *ADH1* mRNA by 2-4 hours. Induction of other anaerobically-induced *Arabidopsis* genes such as pyruvate decarboxylase (*Pdc1*) and sucrose synthase (*ASus1*; Figure 4) shows a similar temporal relationship to AtMYB2 expression.

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The observed root specificity of *ADH1* expression could be a consequence of the tissue-specificity of AtMYB2 expression. If AtMYB2-availability is the limiting factor in *ADH1* expression, and AtMYB2 is only expressed in roots, then *ADH1* will also be expressed only in roots. If AtMYB2 is not expressed in leaves then neither will *ADH1* be. The 25 transactivation results suggest that AtMYB2 is the limiting factor for *ADH1* expression.

We have shown using cycloheximide, that induction of *ADH1* by stress conditions requires protein synthesis, as cycloheximide blocks *ADH1* mRNA accumulation. The fact that AtMYB2 is induced before *ADH1* suggests that synthesis of AtMYB2 could be the protein 30 synthesis required for *ADH1* expression. AtMYB2 mRNA accumulation is not inhibited, but

rather super-induced by cycloheximide treatment. Cycloheximide similarly induces other transcription factors and signal transduction components, such as the maize cold-inducible leucine zipper transcription factor *mlip15*, the calcium-dependent protein kinase *ZmCDPK1* (Berberich and Kusano, 1997), and *HVA22* (Shen *et al.*, 1993). Nuclear run-on experiments  
5 will be required to show whether AtMYB2 accumulation under stress conditions is controlled at the transcriptional or post-transcriptional level and whether the effect of cycloheximide is due to changes in mRNA stability, or to the absence of a repressor. Induction of the maize anaerobic proteins was shown to be preceded by the transient accumulation of two proteins with MW approximately 30 KD (Sachs *et al.*, 1980; MWAtMYB2=27.5 KD). It is therefore  
10 conceivable that AtMYB2 could belong to the maize transition proteins, and that these proteins are required for induction of all the ANPs.

Finally, the third and most important experimental evidence is that the AtMYB2 protein coding region expressed under the control of a strong promoter is able to activate expression  
15 of a reporter gene driven by the *ADH1* promoter. Levels of transactivation seen when AtMYB2 is over-expressed in protoplasts (2-3-fold for biolistics and protoplasts) are similar to those obtained after low oxygen treatment of protoplasts (Figure 6-1 and Figure 6-2). These induction levels were not increased by higher ratios of 35S-AtMYB2: CADH-GUS (data not shown), and were lower than induction levels following low oxygen tension  
20 treatment of roots (5-10-fold at protein level; 20-50-fold at mRNA level). It is possible that the endogenous *ADH1* gene is already somewhat induced after protoplast isolation, and that this leads to higher background activity of the CADH-GUS reporter gene and apparently lower fold induction. *ADH1* gene expression in *Arabidopsis* callus and suspension cultures is generally very high and constitutive (Dolferus *et al.*, 1985). Transient assays using *N.*  
25 *plumbaginifolia* suspension cell protoplasts, gave consistently higher transactivation levels.

Mutation analysis has shown that the GC-motif is equally as crucial as the GT-motif for *ADH1* expression, and AtMYB2 is unable to transactivate *ADH1*-promoter function in the absence of a functional GC-motif (Figure 6-2). In maize, the transcription factor GCBP-1  
30 was shown to interact with the GC-motifs of *Adhl*, and the human general transcription factor

Sp1 was able to bind to the maize *Adhl* GC-motifs (Olive *et al.*, 1991b). It is therefore possible that *ADH1* expression in *Arabidopsis* requires a general transcription factor in addition to AtMYB2, to obtain high levels of expression. The degree of transactivation by AtMYB2 could be limited by other components or processes, such as the requirement for phosphorylation and activation of a signal transduction pathway. It was recently demonstrated that the C terminus of AtMYB2 protein contains a phosphorylation site (Urao *et al.*, 1996), and that truncation of the protein at the C terminus to amino acid 251 leads to higher transactivation levels, indicating that an interaction site with a repressor could also be present in this region (Urao *et al.*, 1996).

10

Vertebrate and yeast Myb transcription factors commonly activate transcription in close association with other factors and to work in a synergistic manner with these factors (Burk *et al.*, 1993; Tice-Baldwin *et al.*, 1989). It has been demonstrated that the maize *Cl myb* and B protein (basic helix-loop-helix factor) interact directly on the maize *Bronze-1* promoter and activate transcription in a cooperative way (Goff *et al.*, 1992). Our data from mutation analysis (Figure 6-2) indicate there may be a similar close association between AtMYB2 and the proteins binding to the GC-motif, and possibly the factor binding to the G-box-1 sequence. In the maize *Adhl* promoter both GT-motifs are closely linked to a GC-motif, while in *Arabidopsis ADH1* the absence of a GC-motif close to the MBS-2 site could explain why this site is not critical for promoter function.

While only one and three Myb genes are known in *Drosophila* and humans respectively (Katzen *et al.*, 1985; Luscher and Eisenmann, 1990), plant Mybs are encoded by large multigene families. An estimated 20-30 Myb genes are present in *Petunia* (Avila *et al.*, 1993). Plant Myb-proteins are involved in the regulation of a variety of processes. Myb transcription factors regulate pigmentation of floral organs, via the regulation of the phenylpropanoid (Sablowski *et al.*, 1994) and flavanoid (Grotewold *et al.*, 1994) biosynthetic pathways. They are also involved in hormone-regulated gene expression and developmental processes, such as the induction of  $\alpha$ -amylase biosynthesis in barley aleurone cells by gibberellic acid (Gubler *et al.*, 1995), trichome formation (Oppenheimer *et al.*, 1991), the

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proliferation of meristematic tissues (Wissenbach *et al.*, 1993), and the regulation of cell shape in petal epidermis cells (Noda *et al.*, 1994). Myb transcription factors are also involved in the regulation of gene expression by environmental stimuli such as induction of anthocyanin biosynthesis by UV-light (Paz-Ares *et al.*, 1987), and in the light-regulation of  
5 an *Arabidopsis Lhcb* gene by phytochrome (Wang *et al.*, 1997). Another desiccation and ABA-induced Myb unrelated to AtMYB2 was found in the resurrection plant *Craterostigma plantagineum* (Iturriaga *et al.*, 1996).

The homology between Myb proteins is restricted to the DNA binding region at the N  
10 terminus. Although plant Mybs can be isolated successfully from cDNA libraries using the animal v-Myb consensus sequence as a probe (Quaedvlieg *et al.*, 1996), the target gene and recognition sequence of many plant Mybs remains unknown. Different plant Mybs show small differences in the recognition sequence (Table II). Maize P Myb, *Antirrhinum* Myb305, potato Myb St1, and especially the *Arabidopsis* ACC 1 factor differ from the  
15 vertebrate Myb consensus sequence (Table II). AtMYB2 can bind to the animal Myb consensus sequence, and activate transcription via this sequence (Urao *et al.*, 1993). Our work has shown that AtMYB2 binds specifically to the GT-motif (MBS-1) and MBS-2 of the *ADHI* promoter. It is able to transactivate the *ADHI* promoter only via the GT-motif, a motif functionally important in a number of anaerobically induced genes (Table I), and which is  
20 functional in either orientation in monocots and dicots (Olive *et al.*, 1991a,b). The GT-motif consensus sequence is 5'-AAACCA-3', and is usually located between positions -300 and -100 relative to the start of transcription (Table I). Conservation of the sequence is generally higher in the 5' end of the central AAC core sequence. This is partly due to the fact that in many cases, such as the maize *Adh1* GT- 1 sequence (Figure 1-1), the GT-motif is flanked  
25 immediately at the 3'-end by a GC-motif (consensus: 5'-GC[G/C]CC-3'; Olive *et al.*, 1991b).

In conclusion, we have identified the transcription factor AtMYB2 as a key factor in the regulation of *ADHI* expression under a variety of environmental stress situations. AtMYB2,  
30 together with a factor binding to the GC-motif, likely to be a general enhancer element, are

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crucial for the attainment of high levels of root-specific expression under low oxygen, and also low temperature and dehydration conditions (Figure 8). The GT- and GC-motifs together are sufficient for low oxygen induction of the *ADH1* promoter, but low temperature, dehydration, and induction by the phytohormone ABA also require the G-box-1 sequence for  
5 high expression levels (Dolferus *et al.*, 1994; de Bruxelles *et al.*, 1996; Figure 8). It has recently been shown that the abscisic acid response complex has a modular nature, and consists of several sequences necessary for obtaining a specific ABA-induced response (Shen *et al.*, 1993; 1996; Shen and Ho, 1995). The combination of the GT-motif and the G-box-1 sequence could be specific for ABA-induced metabolic genes such as the *Arabidopsis ADH1*  
10 gene. Further experiments, using transgenic plants over- and under-expressing AtMYB2 are in progress, with the aim of investigating which genes are induced by AtMYB2, and to verify the similarity between different stress-induced protein patterns and the pattern induced by AtMYB2.

TABLE 1

GENE	SEQUENCE	POSITION	COMMENTS	REFERENCE
Arabidopsis <i>ADH1</i>	5'-CAAA <u>AA</u> CCAAA-3'	-162 to -148	Functionally important.	Dolferus <i>et al.</i> , 1994.
Maize <i>Adh1</i>	5'-GC <del>AA</del> AA <u>AC</u> CCACG-3'	-100 to -114 <sup>(+)</sup>	Functionally important.	Walker <i>et al.</i> , 1987
	5'-GAA <u>AA</u> CCCGGG-3'	-125 to -136 <sup>(+)</sup>		Olive <i>et al.</i> , 1991a,b.
Maize Aldolase	5'-AGAA <u>AA</u> CCAGC-3'	-56 to -72 <sup>(+)</sup>	Located in functionally important region.	Dennis <i>et al.</i> , 1988.
Pea <i>Adh1</i>	5'-ACAA <u>AA</u> CCAAA-3'	-110 to -96	Located in functionally important region.	Llewellyn <i>et al.</i> , 1987.
Maize <i>GapC4</i>	5'-CGAA <u>AA</u> CCAGC-3'	-300 to -285	Located in functionally important region.	Köhler <i>et al.</i> , 1995.
	5'-CGAA <u>AA</u> CCATC-3'	-240 to -227		
CONSENSUS	5'-AA <u>AA</u> CCA-3'			
Arabidopsis <i>PDC1</i>	5'-AAAA <u>AA</u> CCACC-3'	-186 to -172 <sup>(*)</sup>	Homology	Dolferus <i>et al.</i> , in preparation.
	5'-AGAA <u>AA</u> CCAAA-3'	-205 to -191 <sup>(*)</sup>		
Arabidopsis <i>LDH1</i>	5'-AAAA <u>AA</u> CCAAA-3'		Homology	Dolferus <i>et al.</i> , in preparation.

TABLE 1 (cont ')

Arabidopsis <i>ASUS1</i>	5'-TAAAAACCTTG-3' 5'-CAAAACCCAAA-3' 5'-TTTAAACCAAA-3'	-143 to -127 (*) -157 to -142 -220 to -233 (*) -342 to -329	Homology	Martin <i>et al.</i> , 1993.
Maize <i>Adh2-N</i>	5'-AGAAACCCAGG-3'	-131 to -145 (*)	Homology	Dennis <i>et al.</i> , 1985.
Maize Sucrose synthase	5'-CAAAACCCAGA-3'	-161 to -174 (*)	Homology	Werr <i>et al.</i> , 1985.
Wheat <i>Adh1-A</i>	5'-CGAAACCCGGG-3' 5'-GCAAAACCCAAC-3'	-494 to -506 (+,*) -511 to -525 (+,*)	Homology	Mitchell <i>et al.</i> , 1989.
Cotton <i>Adh2</i>	5'-CAAAACCCACG-3' 5'-TAAACCCAGA-3'	-246 to -234 (*) -213 to -201 (*)	Homology	Millar and Dennis, 1996.
Rice <i>Adh2</i>	5'-GGAAACCCACC-3' 5'-GAAACCCGGC-3'	-228 to -213 -209 to -193	Homology	Xie and Wu, 1990.
Strawberry <i>Adh1</i>	5'-CAAAACCCAAACCAA AACC AAAAACCACT-3'	-276 to -310 (+,*)	Homology 4 Tandem Repeats	Wolyn and Jelenkovic, 1990.
Barley <i>Adh2</i>	5'-CACAAACCCAAA-3'	-155 to -171 (*)	Homology	Trick <i>et al.</i> , 1988.
Petunia <i>Adh1</i>	5'-CGAAACCCATT-3'	-176 to -161	Homology	Gregerson <i>et al.</i> , 1991.

TABLE 1 (cont')

Rice <i>Pdcl</i>	5'-CCAAACCTTG-3' 5'-CCAAACCACG-3'	-278 to -292 -277 to -264 (*)	Homology	Hossain <i>et al.</i> , 1996.
OVERALL CONSENSUS SEQUENCE	Position :	-3 -2 -1 A A C C +1 +2 +3		
	% A :	27 52 94		
	% C :	46 14 3	83 35 39	
	% G :	15 31 0	0 24 25	
	% T :	12 3 3	10 28 29	7 14 7
CONSENSUS:	-3 -2 -1	A G A A A A C C	+1 +2 +3	G G A C
	A	A	G	
	A	A	A	
			C	
			C	
rd22 :	T C	T A A C C A C		T
	(Abe <i>et al.</i> , 1997)			

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Asn

**CLAIMS:**

1. A method of modifying the expression of a stress-related gene in a plant cell, tissue or organ or a whole plant, said method at least comprising the step of expressing therein a MYB2 protein or a functionally-equivalent homologue, analogue or derivative thereof for a time and under conditions sufficient for expression of said stress-related gene to be increased, reduced or otherwise altered.
2. The method according to claim 1, wherein the expression of the stress-related gene is increased by expression of the MYB2 protein.
3. The method according to claims 1 or 2, wherein the MYB2 protein comprises an amino acid sequence set forth in <400>2 or <400>4 or a functionally-equivalent homologue, analogue or derivative thereof which is at least about 70% identical thereto.
4. The method according to any one of claims 1 to 3, wherein the stress-related gene comprises a promoter having at least one copy of a *cis*-acting regulatory nucleotide sequence designated "MBS-1 motif" as hereinbefore defined or a complementary nucleotide sequence thereto.
5. The method according to any one of claims 1 to 4, wherein the stress-related gene comprises a promoter having at least one copy of a *cis*-acting regulatory nucleotide sequence designated "GC-motif" as hereinbefore defined or a complementary nucleotide sequence thereto.
6. The method according to any one of claims 1 to 5, wherein the stress-related gene comprises a promoter having at least one copy of a *cis*-acting regulatory nucleotide sequence designated "G-box-1" as hereinbefore defined or a complementary nucleotide sequence thereto.

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7. The method according to any one of claims 1 to 6, wherein the stress-related gene comprises a promoter having at least one copy of a *cis*-acting regulatory nucleotide sequence designated "G-box-2" as hereinbefore defined or a complementary nucleotide sequence thereto.
8. The method according to any one of claims 4 to 7, wherein the stress-related gene encodes an enzyme selected from the list comprising alcohol dehydrogenase, aldolase, glyceraldehyde phosphate dehydrogenase, pyruvate decarboxylase, sucrose synthase and lactate dehydrogenase.
9. The method according to claim 8, wherein the stress-related gene encodes alcohol dehydrogenase.
10. The method according to any one of claims 1 to 3, wherein the stress-related gene is induced in response to anaerobic stress.
11. The method according to claim 10, wherein the stress-related gene encodes alcohol dehydrogenase.
12. The method according to claim 10, wherein the stress-related gene encodes pyruvate decarboxylase.
13. The method according to claim 10, wherein the stress-related gene encodes an enzyme selected from the list comprising sucrose synthase, phosphoglucomutase, phosphoglucose isomerase, fructose-1,6-diphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, enolase and alanine amino transferase.
14. The method according to any one of claims 1 to 3, wherein the stress-related gene is induced in response to dehydration or water loss or reduced turgor in the plant.

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15. The method according to claim 14, wherein the stress-related gene encodes alcohol dehydrogenase.
16. The method according to any one of claims 1 to 3, wherein the stress-related gene is induced in response to cold stress.
17. The method according to claim 16, wherein the stress-related gene encodes alcohol dehydrogenase.
18. The method according to any one of claims 1 to 3, wherein the stress-related gene is induced by an environmental stress selected from the list comprising flooding stress, heat stress, drought stress and salt stress.
19. The method according to any one of claims 1 to 3, wherein the stress-related gene is induced by the application of abscisic acid.
20. The method according to claim 19, wherein the stress-related gene encodes alcohol dehydrogenase.
21. The method according to any one of claims 1 to 20 comprising the additional first step of introducing into the plant cell, tissue, organ or whole plant an isolated nucleic acid molecule which encodes said MYB2 protein or functionally-equivalent homologue, analogue or derivative thereof.
22. The method according to claim 21 wherein the isolated nucleic acid molecule comprises the nucleotide sequence set forth in <400>1 or <400>3 or a homologue, analogue or derivative that is at least about 75% identical thereto or a complementary nucleotide sequence thereof.
23. The method according to claims 21 or 22, wherein the isolated nucleotide sequence

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is operably linked to a promoter sequence that is operable in the plant cell, tissue, organ or whole plant.

24. The method according to claim 23 wherein the promoter is the CaMV 35S promoter sequence.

25. The method according to claim 23 wherein the promoter is a glucocorticoid-inducible promoter sequence.

26. A genetic construct which comprises a nucleotide sequence set forth in <400> 1 or <400> 3 or a homologue, analogue or derivative thereof which encodes a functional MYB2 polypeptide and is at least about 75% identical to said nucleotide sequence, wherein said genetic construct expresses a MYB2 protein in a plant cell, tissue, organ or whole plant to modify the expression of a stress-related gene therein.

27. The genetic construct according to claim 26, wherein the nucleotide sequence is placed operably under the control of a promoter sequence that is operable in the plant cell, tissue, organ or whole plant.

28. The genetic construct according to claim 27 wherein the promoter is the CaMV 35S promoter sequence.

29. The genetic construct according to claim 27 wherein the promoter is a glucocorticoid-inducible promoter sequence.

30. A method of producing a stress-tolerant plant comprising the steps of transforming plant tissue or cells with the genetic construct according to any one of claims 26 to 29, regenerating said tissue or cells into a whole plant and expressing the MYB2 protein encoded by said genetic construct therein for a time and under conditions sufficient for expression of a stress-related gene in said plant to be increased, reduced or otherwise altered.

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31. A method of producing a stress-tolerant plant comprising at least the step of expressing a MYB2 protein or a homologue, analogue or derivative thereof in said plant or a cell, tissue or organ thereof for a time and under conditions sufficient for expression of a stress-related gene in said plant to be increased, reduced or otherwise altered.

32. The method according to claims 30 or 31 wherein the stress-related gene in said plant is increased.

33. The method according to any one of claims 30 to 32, wherein the MYB2 protein comprises an amino acid sequence set forth in <400>2 or <400>4 or a functionally-equivalent homologue, analogue or derivative thereof which is at least about 70% identical thereto.

34. The method according to any one of claims 30 to 33, wherein the stress-related gene comprises a promoter having at least one copy of a *cis*-acting regulatory nucleotide sequence designated "MBS-1 motif" as hereinbefore defined or a complementary nucleotide sequence thereto.

35. The method according to any one of claims 30 to 34, wherein the stress-related gene comprises a promoter having at least one copy of a *cis*-acting regulatory nucleotide sequence designated "GC-motif" as hereinbefore defined or a complementary nucleotide sequence thereto.

36. The method according to any one of claims 30 to 35, wherein the stress-related gene comprises a promoter having at least one copy of a *cis*-acting regulatory nucleotide sequence designated "G-box-1" as hereinbefore defined or a complementary nucleotide sequence thereto.

37. The method according to any one of claims 30 to 36, wherein the stress-related gene comprises a promoter having at least one copy of a *cis*-acting regulatory nucleotide sequence

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designated "G-box-2" as hereinbefore defined or a complementary nucleotide sequence thereto.

38. The method according to any one of claims 30 to 33, wherein the stress-related gene encodes an enzyme selected from the list comprising alcohol dehydrogenase, aldolase, pyruvate decarboxylase, sucrose synthase, lactate dehydrogenase, phosphoglucose isomerase, fructose-1,6-diphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, enolase and alanine amino transferase.

39. The method according to claim 38, wherein the stress-related gene encodes alcohol dehydrogenase.

40. The method according to claim 38, wherein the stress-related gene encodes pyruvate decarboxylase.

41. The method according to any one of claims 30 to 40, wherein the stress to which the plant is made tolerant comprises anaerobic stress.

42. The method according to any one of claims 30 to 40, wherein the stress to which the plant is made tolerant comprises dehydration or water loss or reduced turgor in the plant.

43. The method according to any one of claims 30 to 40, wherein the stress to which the plant is made tolerant comprises cold stress.

44. The method according to any one of claims 30 to 40, wherein the stress to which the plant is made tolerant comprises an environmental stress selected from the list comprising flooding stress, heat stress, drought stress and salt stress.

45. A transgenic plant produced by the method according to any one of claims 30 to 44.

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46. A plant having modified expression of a stress-related gene, wherein said plant is produced by the method according to any one of claims 1 to 25.
47. A cell, tissue or organ derived from the plant according to claims 45 or 46, wherein said cell, tissue or organ has modified expression of a stress-related gene.
48. A plant cell, tissue or organ other than a whole plant which exhibits modified expression of a stress-related gene by virtue of the expression therein of a MYB2 protein.

**MYB CONSENSUS:**      **ANIMAL:**      [CT]AACGG

**PLANT P-MYB:**      CCT/A/AACC

### FIGURE 1-1

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OLIGO :	SEQUENCE :
MBS-1	5'-GGGAA <u>AA</u> CCAAATCCG-3'
ΔMBS-1/1	5'-GGGA <u>ACCC</u> CAAATCCG-3'
ΔMBS-1/2	5'-GGGAAG <u>AC</u> CAAATCCG-3'
MBS-2	5'-GGCTAGCA <u>AC</u> GCCAAGTGG-3'
ΔMBS-2	5'-GGCTAGCC <u>CC</u> GCCAAGTGG-3'
4 X MBS-1	4 X [5'-GGGAA <u>AA</u> CCAAATCCG-3']
ΔGT	5'-GGCCTCTAGACCTAC-3'
ΔGC	5'-TACGAGAAATAGT-3'
ΔGBOX1	5'-AAATAACATGTTTCACG-3'
ΔGBOX2	5'-GCAACTAACATGTTCCCGAG-3'
GBS	5'-GGCCGATAACAACCTCCGG-3'

FIGURE 1-2

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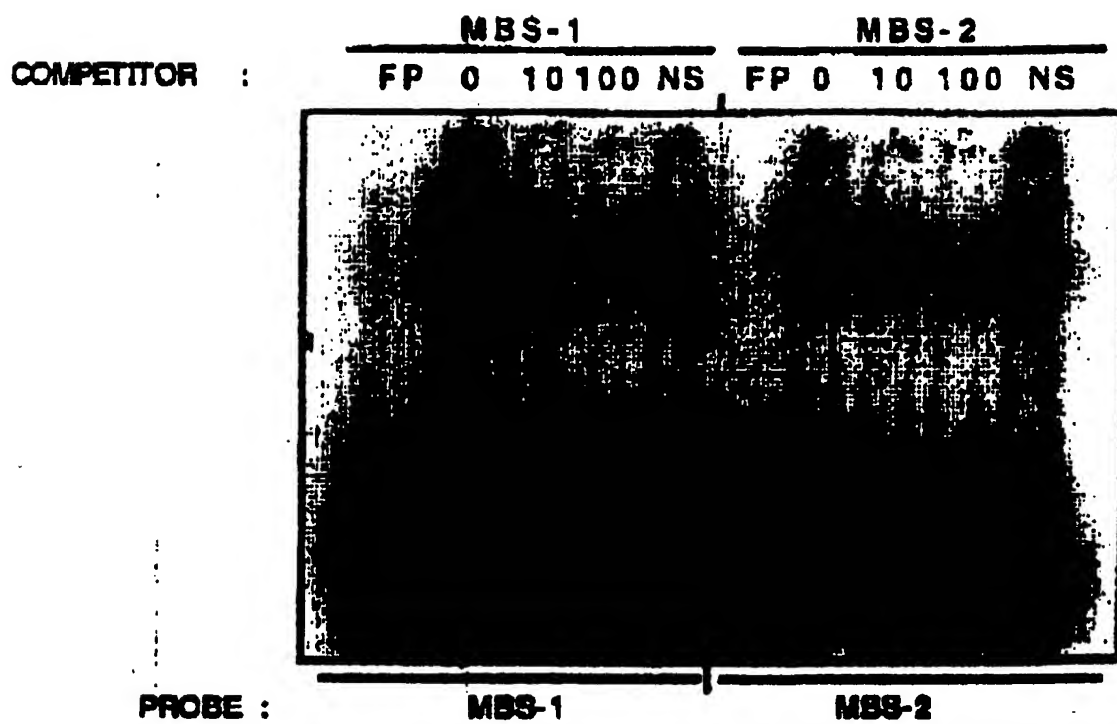


FIGURE 2-1

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COMPETITOR : FP 0 10 100 FP 10 100 200 500 NS



PROBE :

MBS-1

4 X MBS-1

FIGURE 2-2

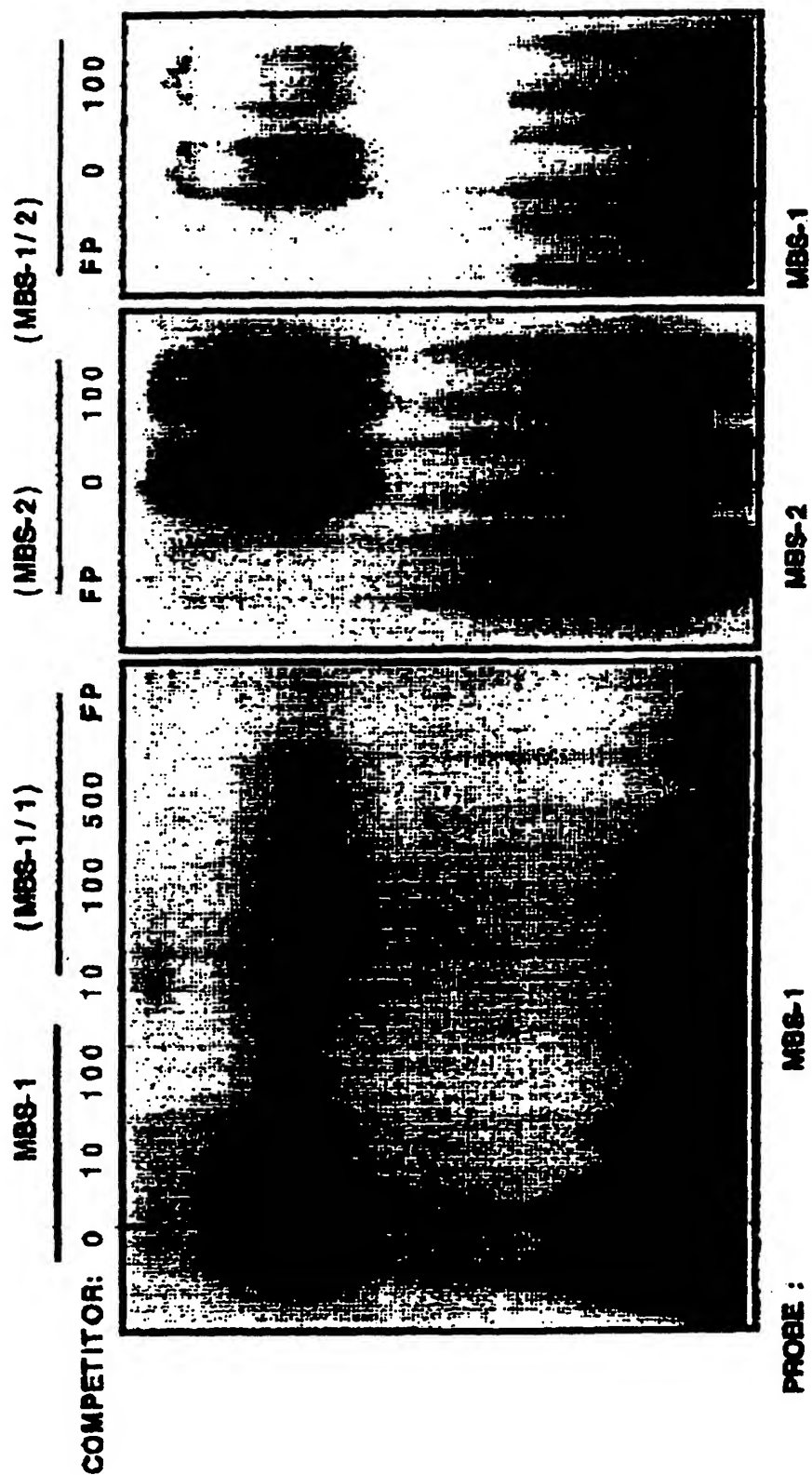


FIGURE 2-3

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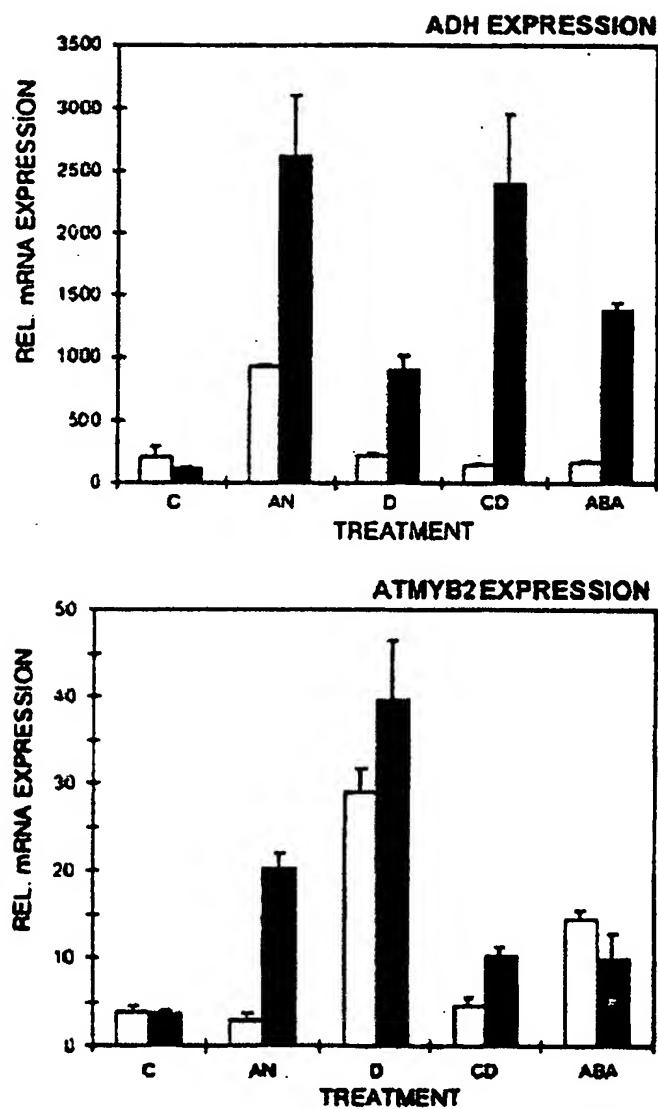


FIGURE 3-1

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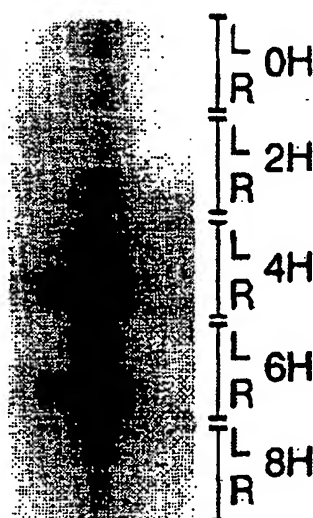


FIGURE 3-2

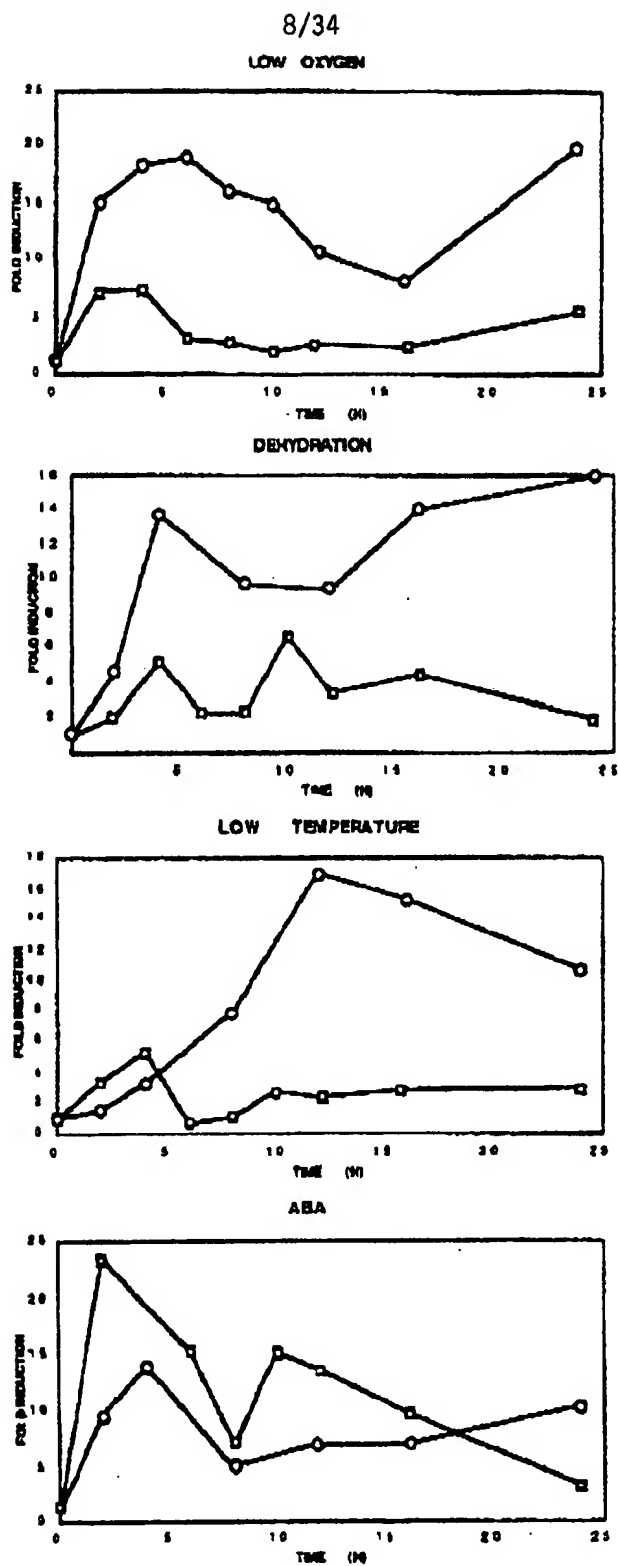


FIGURE 3-3

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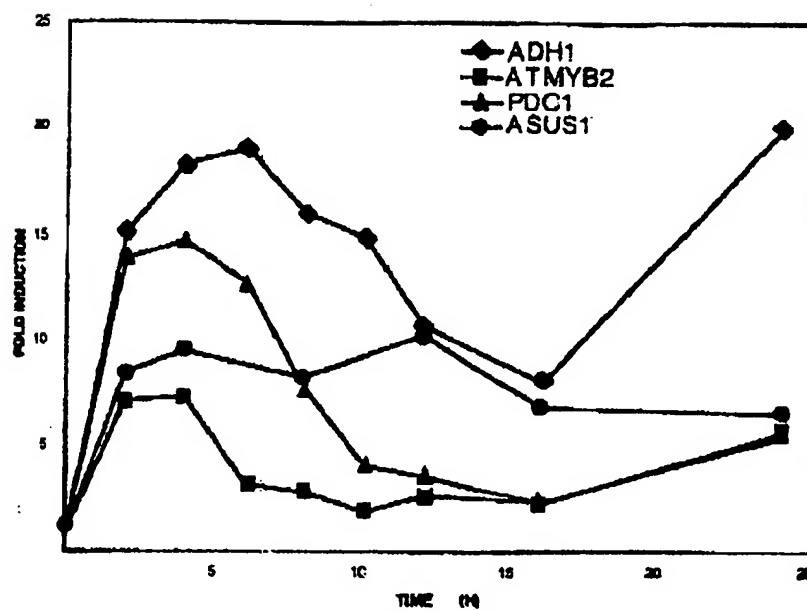


FIGURE 4

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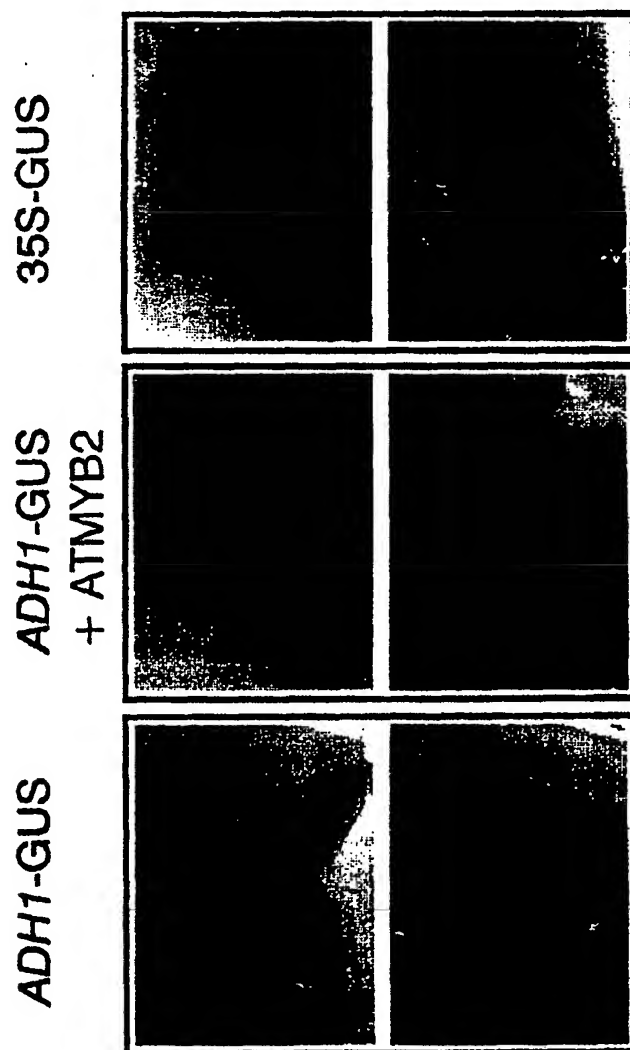
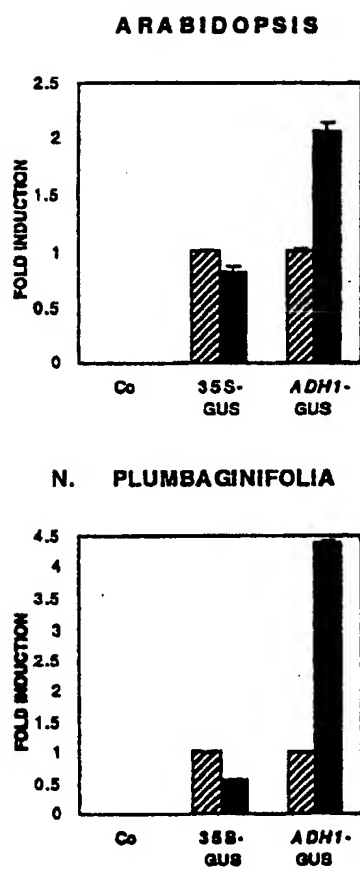


FIGURE 5

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**FIGURE 6-1**

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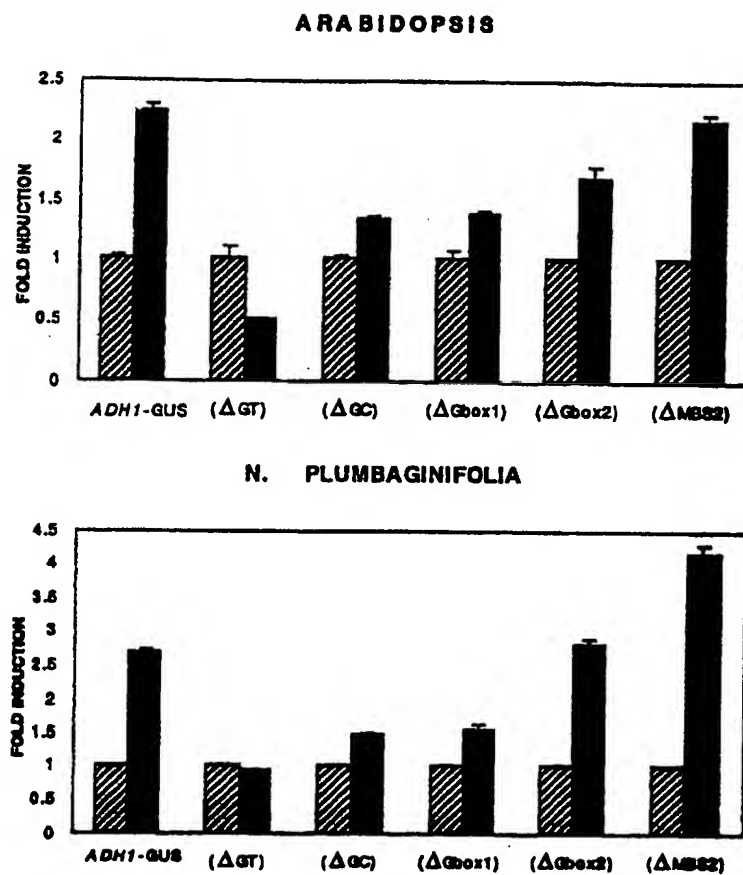


FIGURE 6-2

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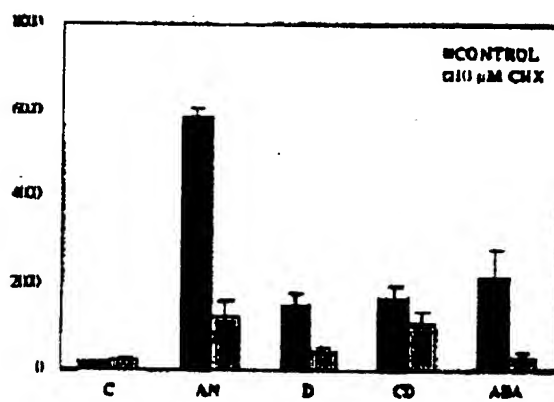


FIGURE 7-1

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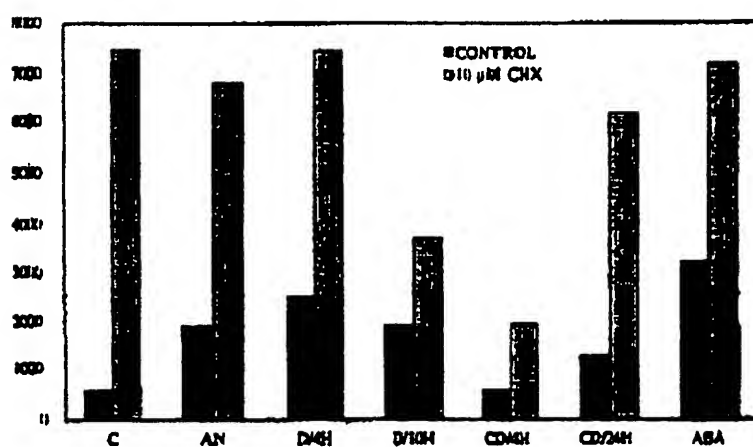


FIGURE 7-2

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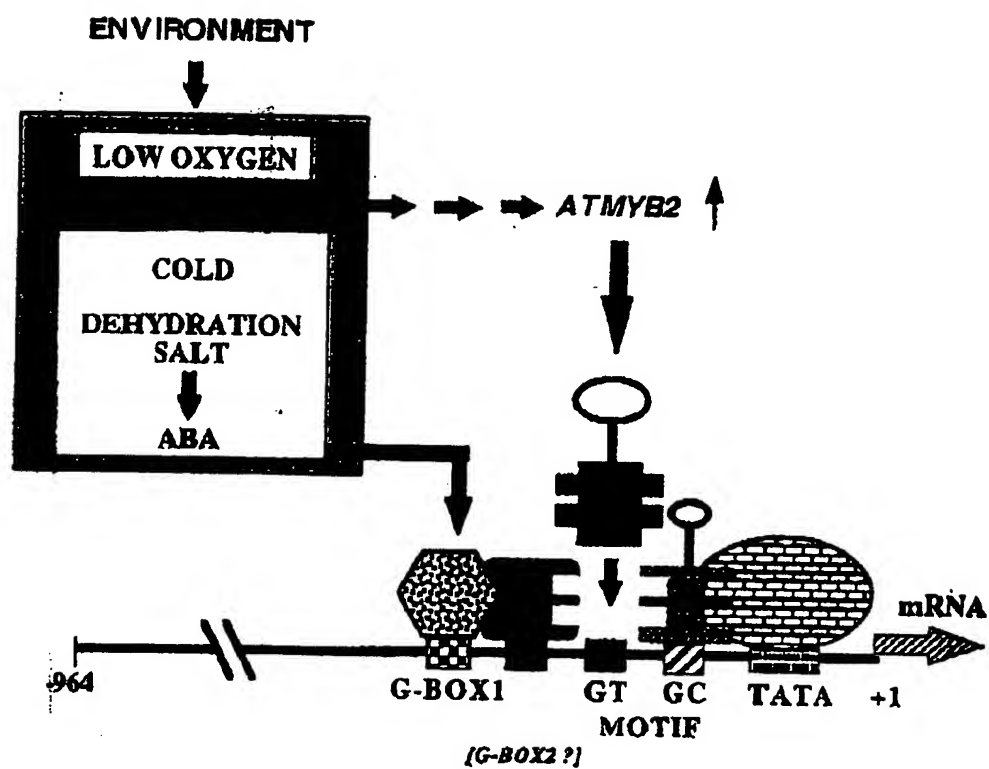
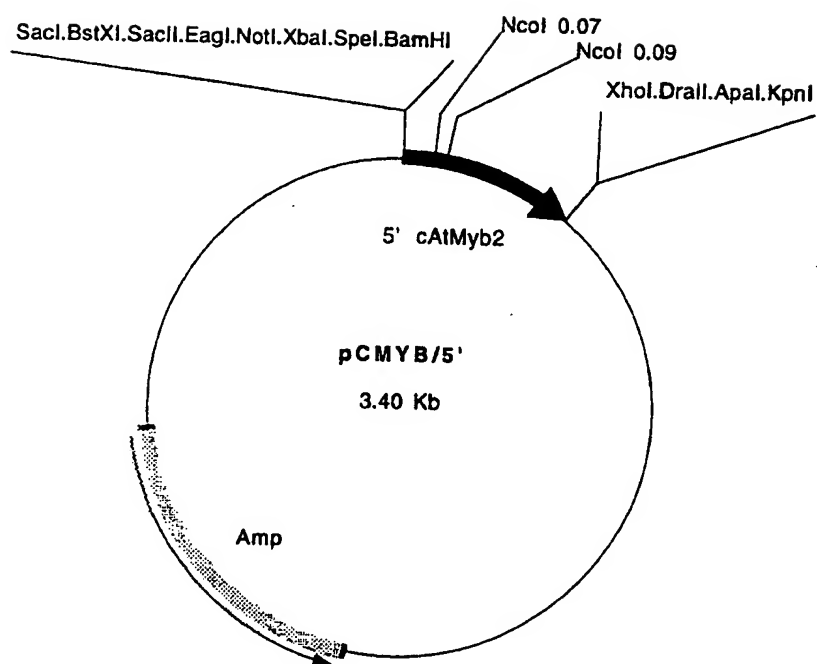


FIGURE 8

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**FIGURE 9**

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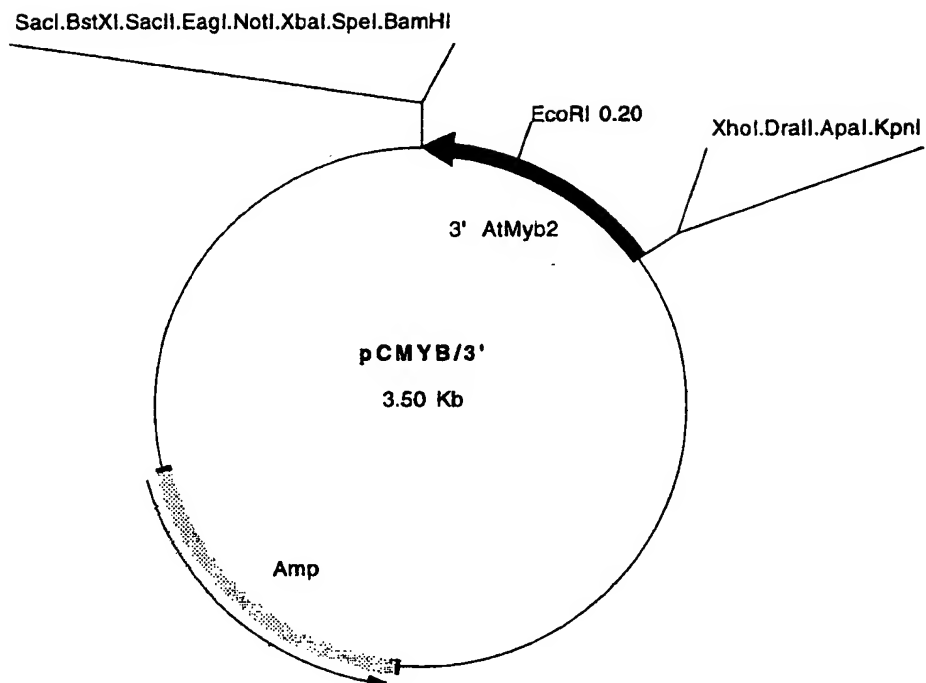


FIGURE 10

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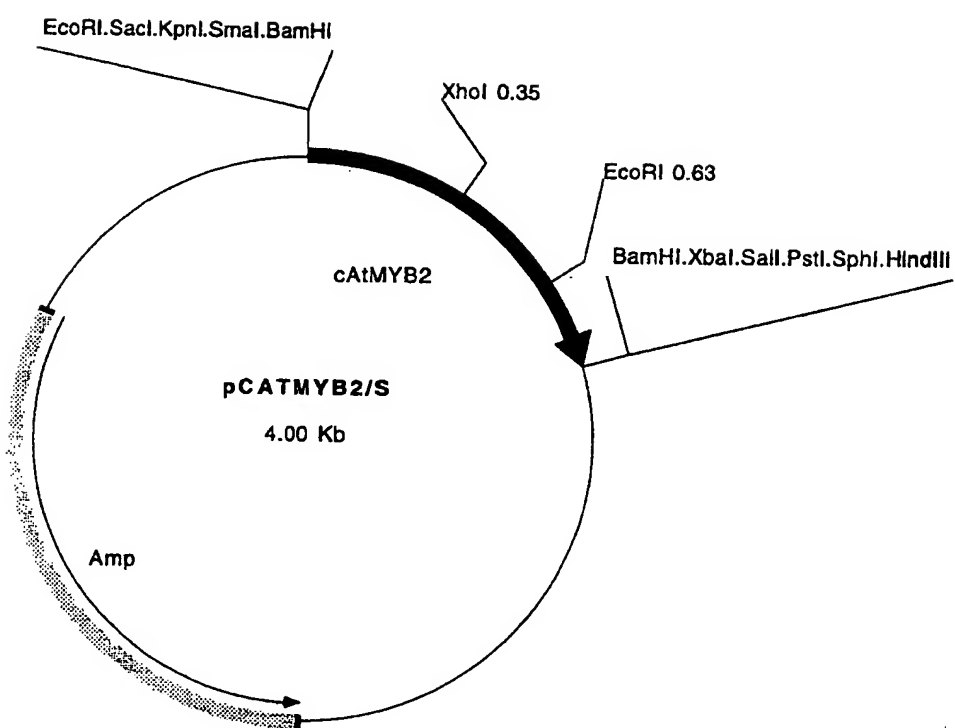
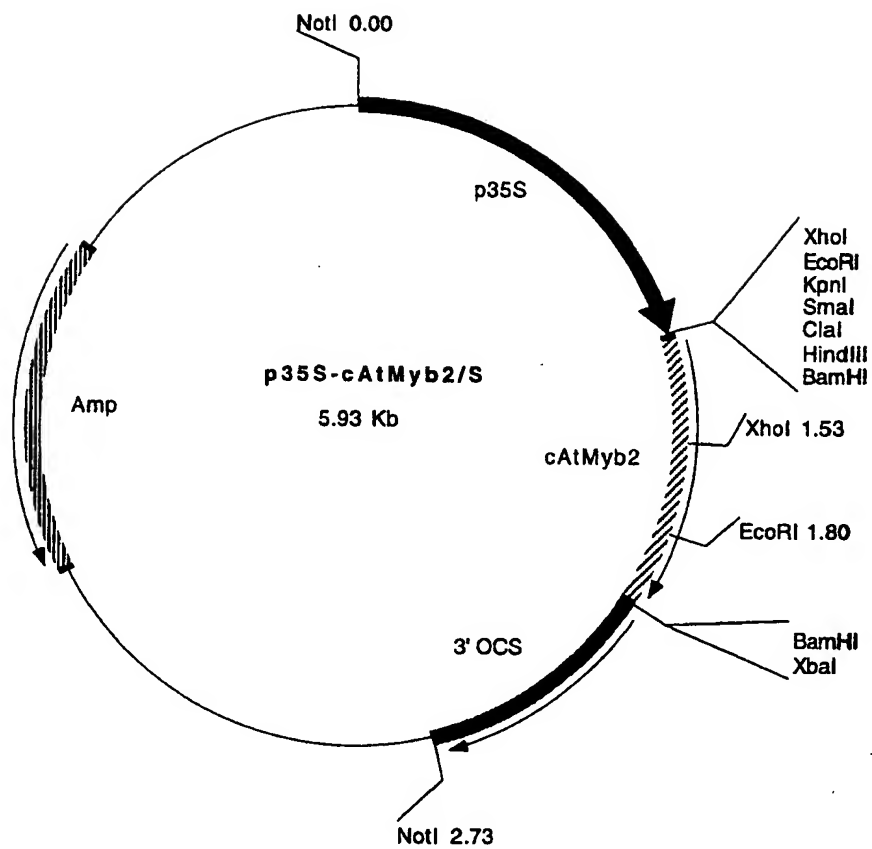
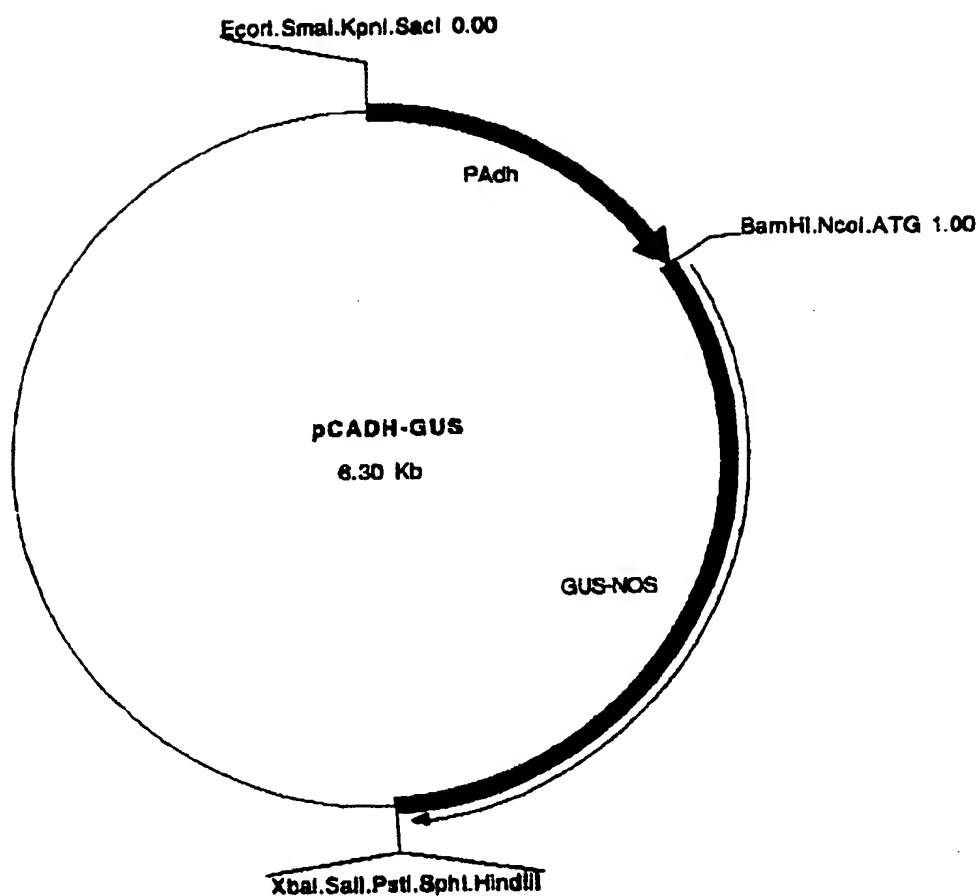


FIGURE 11

**FIGURE 12**



**FIGURE 13**

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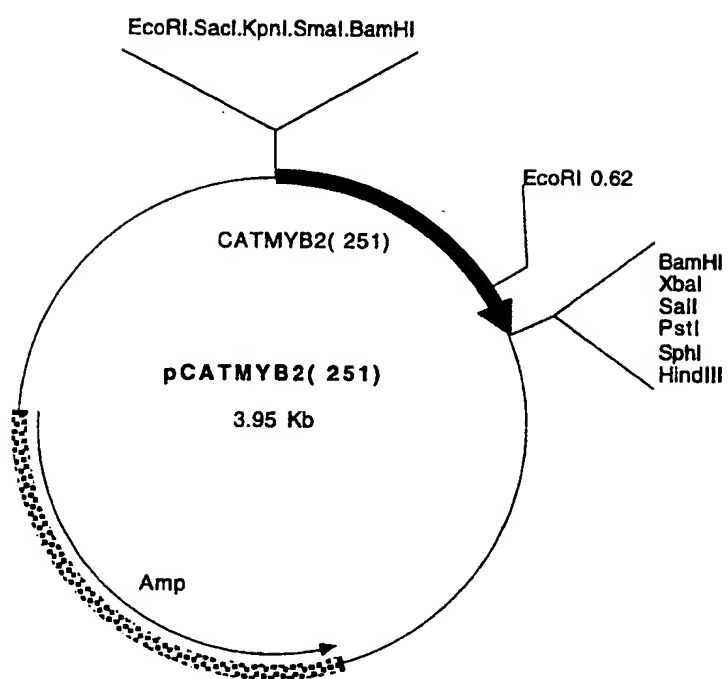


FIGURE 14

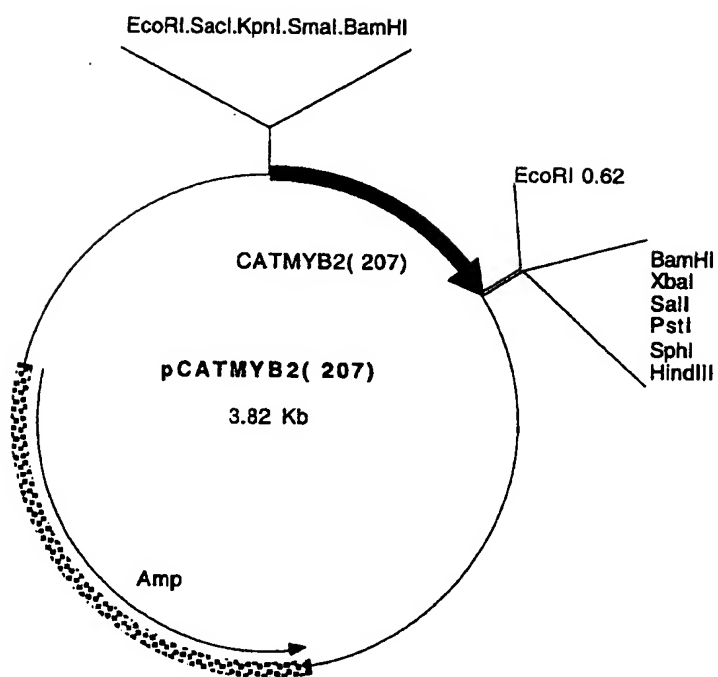
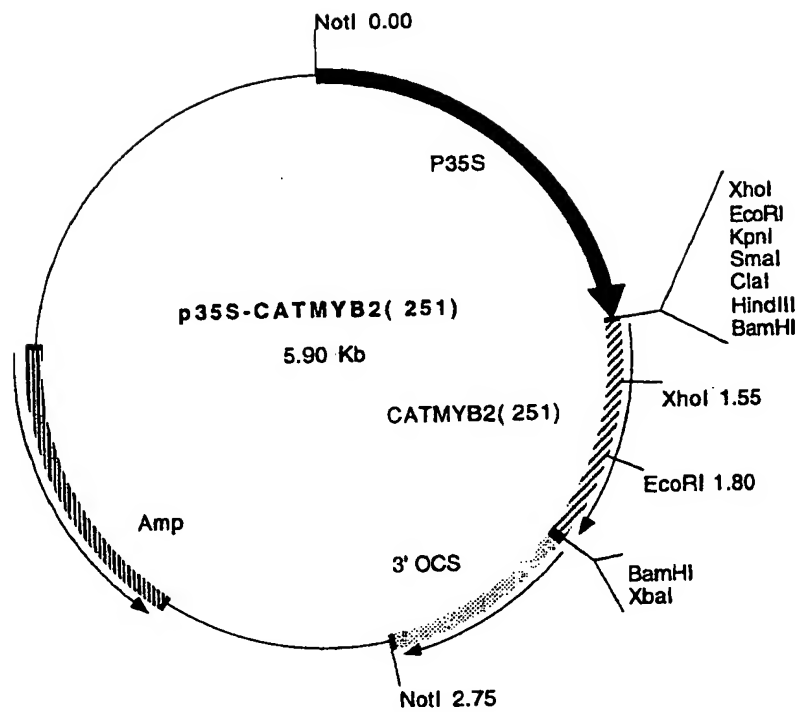


FIGURE 15

**FIGURE 16**

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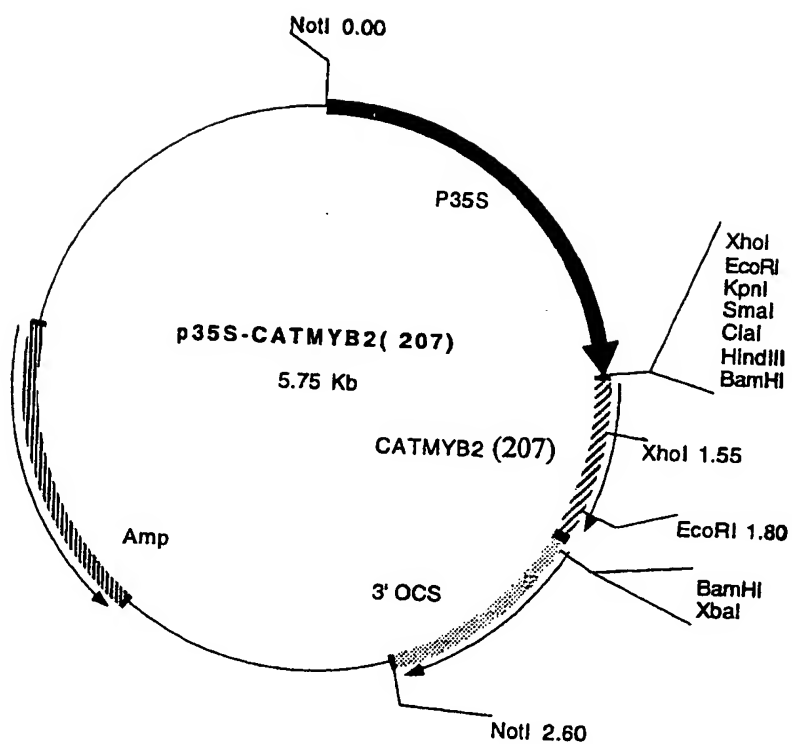


FIGURE 17

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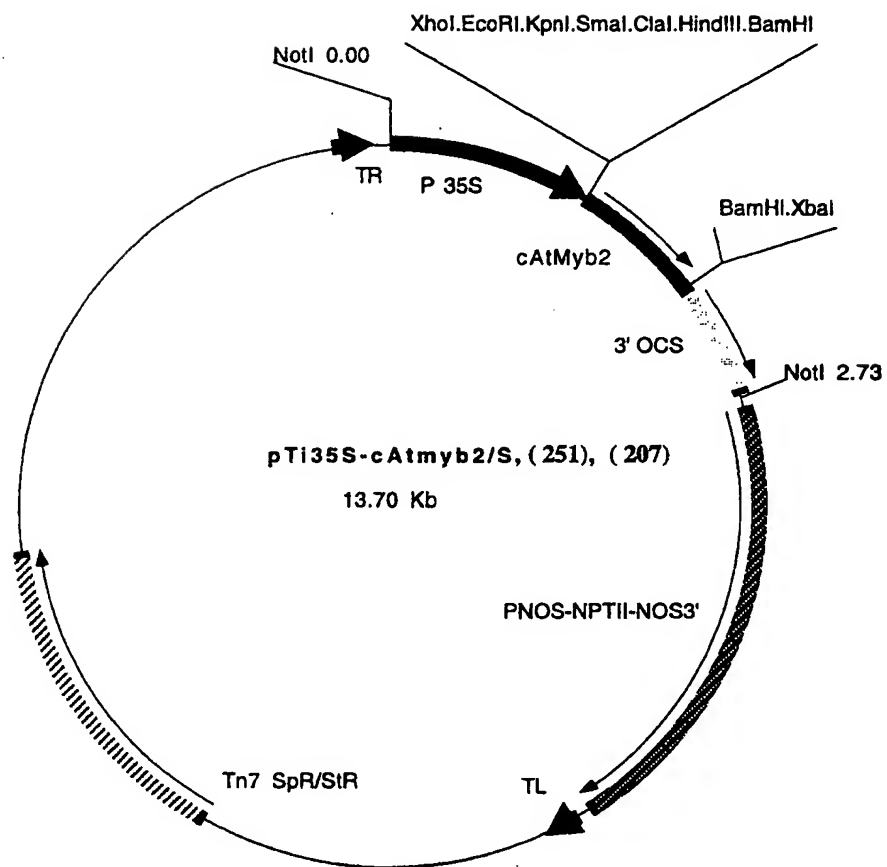


FIGURE 18

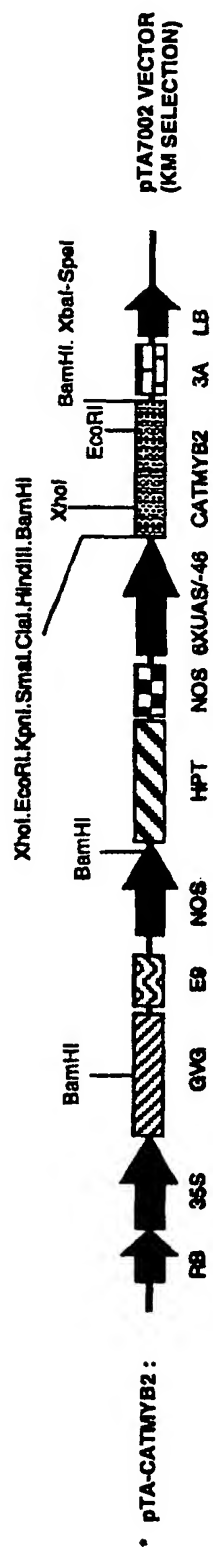


FIGURE 19-1

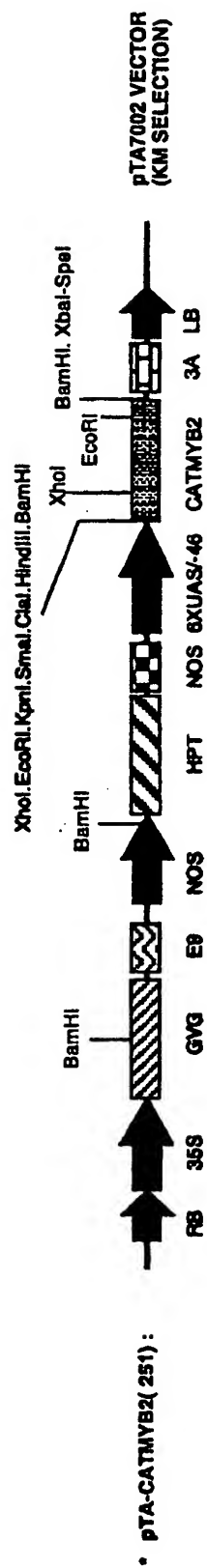


FIGURE 19-2

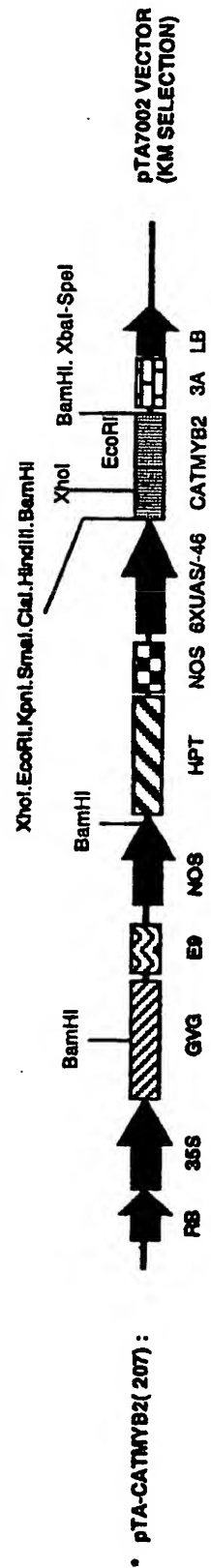
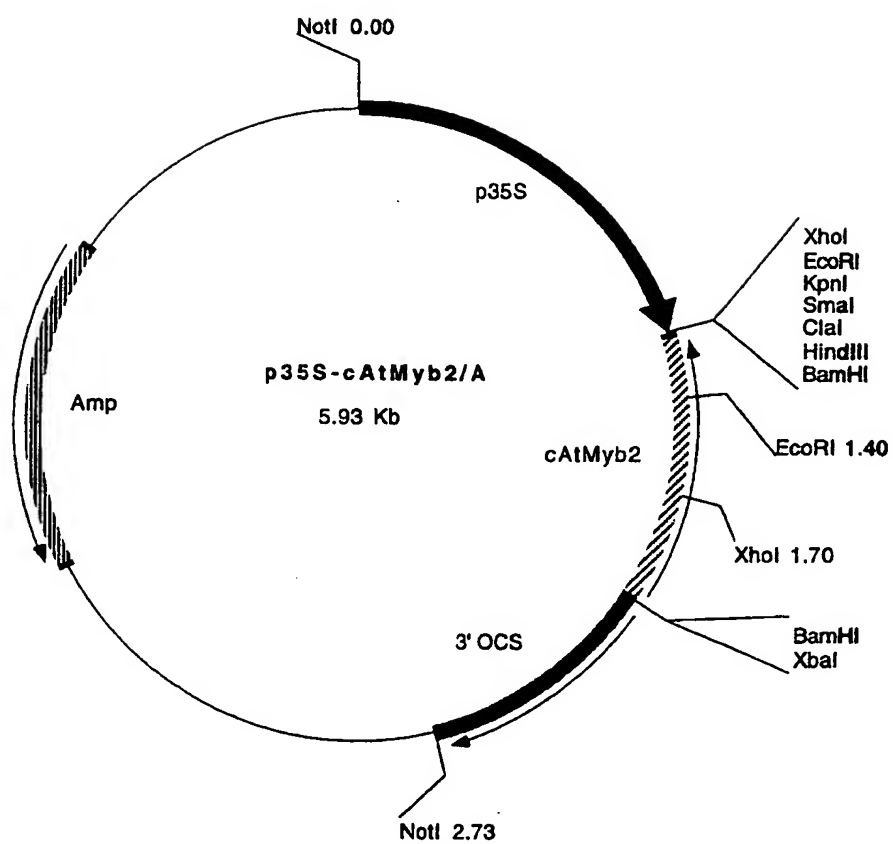
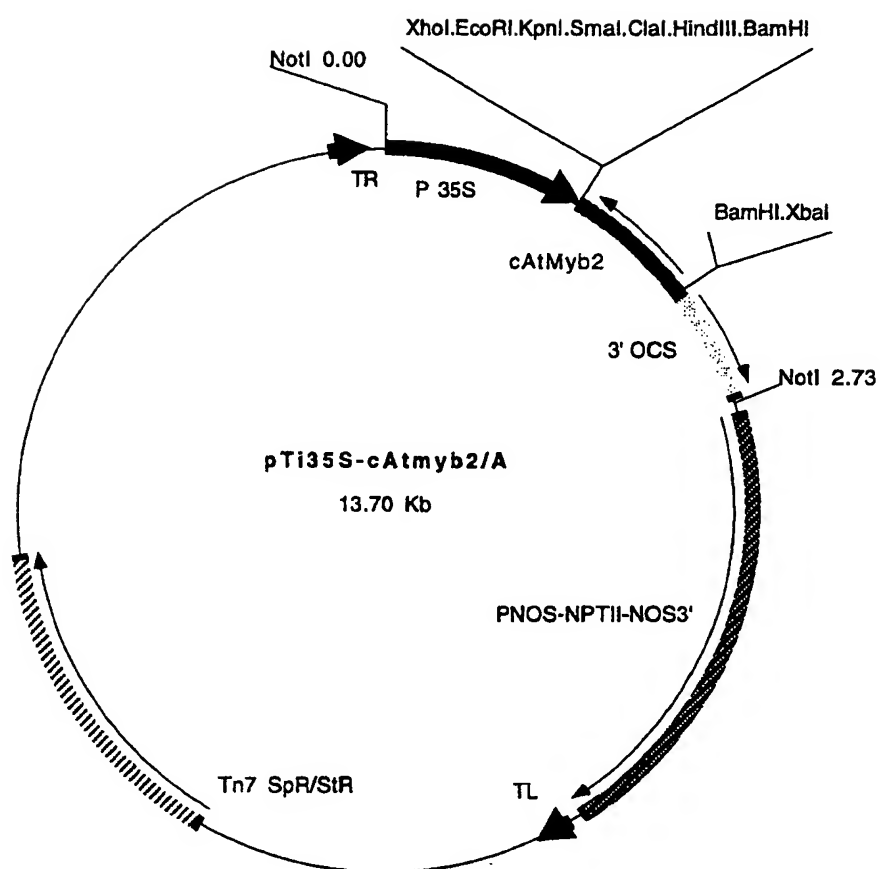


FIGURE 19-3

**FIGURE 20**

**FIGURE 21**

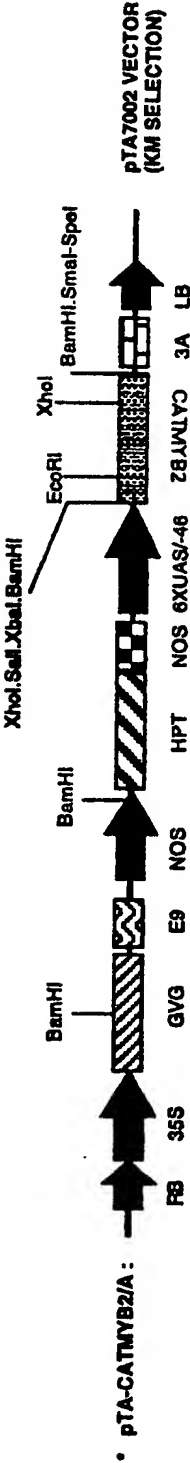
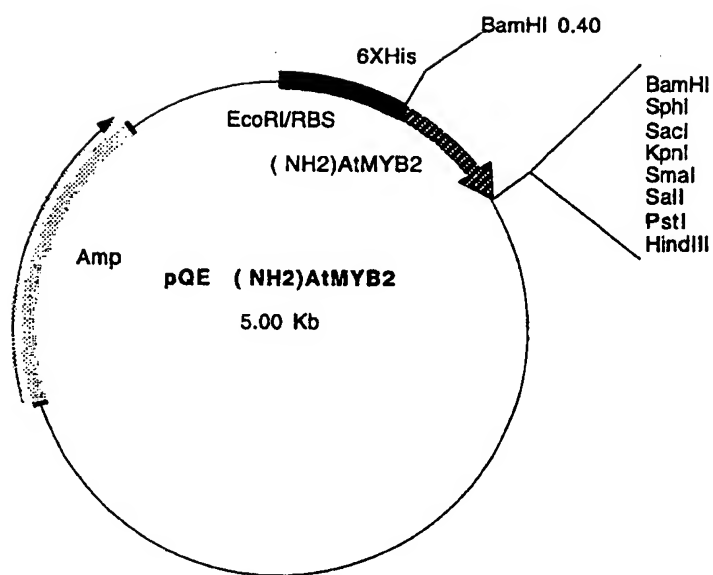
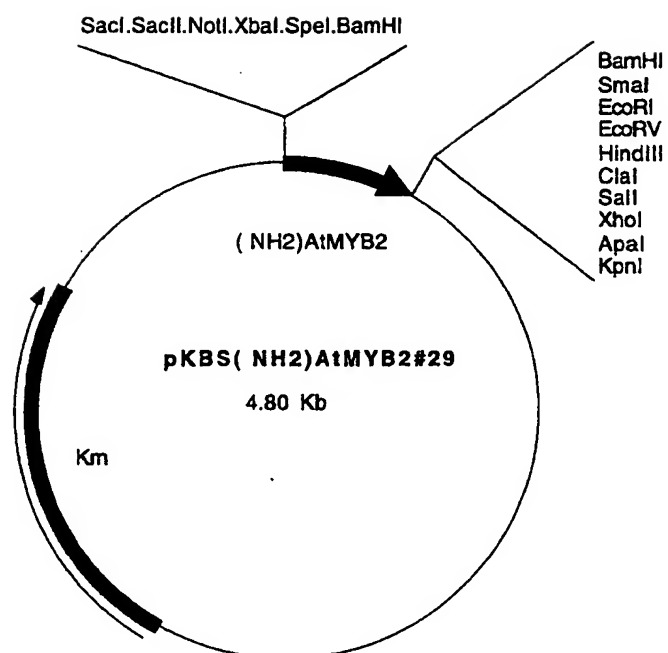


FIGURE 22

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**FIGURE 23**

**FIGURE 24**

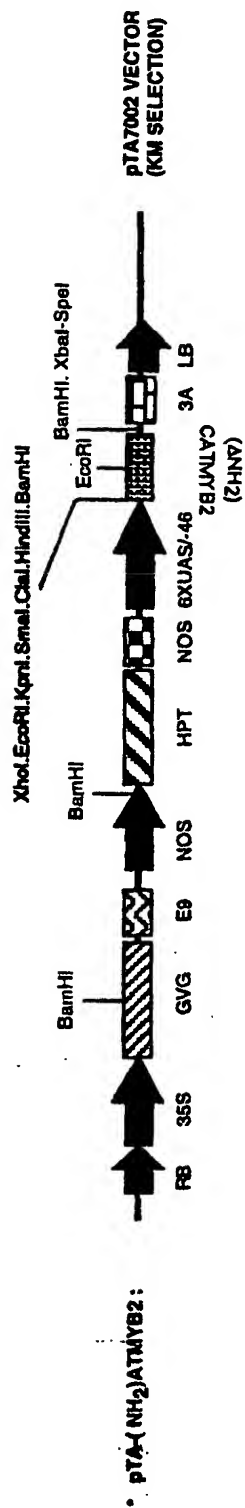


FIGURE 25

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/AU 98/00812

## A. CLASSIFICATION OF SUBJECT MATTER

Int Cl<sup>6</sup>: C12N 15/29, 15/82; A01H 1/00, 5/00.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
See key words in electronic data box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Japio, USPM, Medline and GENE BANK EMBL SWISS-PROT, PIR See key words in electronic data box below.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
WPAT (Derwent), Japio and USPM: Keywords (a) Atmyb##; (b) myb## and (dehydrat: or desiccate: or stress: or low oxygen). Chemical Abstracts and Medline: strategies (a); (b) and (c) myb? and see continuation sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	Hoeren F. U. et al, "Evidence for a role for ATMYB2 in the induction of Arabidopsis alcohol dehydrogenase gene (ADH1) by low oxygen" Genetics 1998 149 page 479-490. See the whole document.	1-48
PX	Kirik V. et al, "Ectopic expression of a novel MYB gene modifies the architecture of the Arabidopsis inflorescence" Plant J. 1998 13 pages 729-742. See especially pages 737-739.	1,2
PX	Abe H. et al, "Role of arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression" Plant Cell 1997 9 pages 1859-1868. See especially page 1865.	1-3

☒ Further documents are listed in the continuation of Box C

☐ See patent family annex

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance  
"E" earlier application or patent but published on or after the international filing date  
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
"O" document referring to an oral disclosure, use, exhibition or other means  
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art  
"&" document member of the same patent family

Date of the actual completion of the international search  
19 November 1998

Date of mailing of the international search report  
23 NOV 1998

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JH CHAN  
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00812

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
X	Urao T. et al, "A transcriptional activation domain of ATMYB2, a drought-inducible Arabidopsis Myb-related protein." Plant J. 1996 10 pages 1145-1148. See especially pages 1145 -1146.	1-3, and 26-29
X	Iturriaga G. et al, "A family of novel myb-related genes from the resurrection plant <i>Craterostigma plantagineum</i> are specifically expressed in callus and roots in response to ABA or desiccation". Plant Molecular Biology 32 pages 707-716 (1996). See especially pages 713-714.	1-3, 18, 19 and 26.
X	Yamaguchi-Schinozaki K. et al, "Regulation of genes that are induced by drought stress in <i>Arabidopsis thaliana</i> " J. Plant Res. 108 pages 127-137 (1996). See especially page 133 onwards.	1-3.
X	Urao T. et al "An arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence." Plant Cell 1993 5 pages 1529-1539. See the whole document.	1-3, 18, 19, 21-23 26 and 17.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00812

B x B: Elcetric data bas box: ntinued:

and (antirrhinum or snapdragon? or pisum or pea? or gossypium or craterostigma or cotton). Sequences id 2 and 4.